

**Regulation of flagellin glycosylation genes in  
*Campylobacter jejuni*: influence of NssR, the  
nitrosative stress response regulator**

**by**

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## Abstract

*Campylobacter jejuni* is one of the leading causes of food borne illness worldwide; the main cause of infection is the consumption of undercooked, contaminated poultry. Motility, mediated by single polar flagella, is necessary for *Campylobacter* virulence. The flagellin proteins are extensively glycosylated in *C. jejuni* with pseudaminic acid and related derivatives; glycosylation is required for flagella filament formation. The flagellin glycosylation locus in *C. jejuni* NCTC11168 comprises 50 genes, 50% are potentially involved in glycan biosynthesis and others are hypothetical; the regulatory mechanism(s) of flagellin glycosylation remain unclear. The response to nitric oxide stress in *C. jejuni* is regulated by NssR, which also regulates a small number of genes in the flagellin glycosylation locus; an *nssR* mutant has defective motility. This work aims to elucidate the mechanisms by which NssR regulates these genes and investigate the consequences of *nssR* mutation. Transcriptional organisation of the affected genes was deduced and chromatin immunoprecipitation (ChIP) used to map NssR binding to six potential locations in the region. Methods to measure bacterial motility were assessed and used to document the defective motility of the *nssR* mutant, which was found to be due to truncated, sometimes asymmetric, flagella. Finally, mass spectrometry was used to examine flagellin glycosylation in all strains.

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# Chapter I: Introduction

## 1.1 A brief history of *Campylobacter*

Whilst campylobacters were not widely recognised as human pathogens until the 1970's, they were probably first observed in 1886 by German bacteriologist Theodor Escherich, who described spiral bacteria in the colons of infants who had died from enteric disease (Escherich, 1886). Escherich was unable to cultivate these bacteria on solid media and, despite observing that these organisms were more frequently found in stool specimens from infants with enteric disease, thought that they played no aetiological role (Kist, 1985). In the following years several reports were made of non-culturable bacteria with similar characteristics, but the lack of suitable selective media made further investigation difficult. However, in 1909, two veterinary surgeons reported the discovery of an unknown vibrio-like organism, which could be frequently isolated from the aborted fetuses of ewes (McFadyean and Stockman, 1913). In 1919 Smith and Taylor isolated a bacterium they described as a 'spirillum' from aborted bovine fetuses and concluded that this bacterium was identical to the vibrio-like bacterium of McFadyean and Stockman; consequently the name '*Vibrio fetus*' was proposed for these organisms. In 1931, Jones *et al.* connected winter dysentery in calves with a '*Vibrio*' they called '*Vibrio jejuni*'; in 1944 a similar organism associated with dysentery in swine was reported (Doyle, 1944).

Whilst the role of campylobacters as veterinary pathogens was already quite well established, the first well documented instance of human *Campylobacter* infection did not occur until 1938. This involved a milk-borne outbreak of enteritis, while microscopy only positively identified '*V. jejuni*' in 10% of the cases, organisms resembling '*V. jejuni*' were



grown in liquid cultures of the blood of 3.5% of those infected (Levy, 1946). In 1963 the genus *Vibrio* underwent a major taxonomic change. Sebald and Véron (1963) proposed *Campylobacter* (Greek, campylo: 'curved') as a new genus, to incorporate those 'vibrios' already known to differ from the rest of the genus, most notably by low DNA base composition and microaerophilic growth requirement. Immediately after its inception the genus *Campylobacter* contained two species, '*C. fetus*' and '*C. bubulus*'. The number of species increased dramatically in the following years, especially following the development of a filtration technique by Dekeyser *et al.* in 1972 that allowed the first isolation of *Campylobacter* from faeces. The development of a selective medium by Skirrow (1977) was another revolution in *Campylobacter* research, as was the increased availability and sophistication of equipment and methods for generating microaerobic environments. The relative simplicity of the isolation procedure allowed the isolation of *Campylobacter* and *Campylobacter*-like organisms by less specialised diagnostic microbiology laboratories and, for the first time, allowed the importance of campylobacters as infectious agents in humans to be identified.

## 1.2 Taxonomy

*Campylobacter* is included, together with the genera *Arcobacter* and *Sulfurospirillum* in the family *Campylobacteraceae* (Vandamme *et al.*, 1991; Stolz *et al.*, 1999), which has been shown by partial 23S rRNA analysis to be part of the delta-epsilon subdivision of *Proteobacteria* (Trust *et al.*, 1994). Following the above mentioned development of a selective medium by Skirrow (1977), interest in the genus increased considerably as the importance of *Campylobacter* in human disease began to be realised; this led to the description of many new species of campylobacters from varying sources. The greater

number of species and the ever-increasing sophistication of methods used to classify bacteria lead to re-arrangement within the genus itself. In 1987 studies of 16S rRNA gene sequence divergence in *Campylobacter* by Lau *et al.* (1987) demonstrated the diversity of the genus as then defined; the genus *Helicobacter* was created by Goodwin *et al.* (1989) by the reassignment of '*C. pylori*' and '*C. mustelae*', as these species were seen to possess sheathed flagella, differing cellular fatty acid composition and a divergence in 16S rRNA sequences from the other members of the *Campylobacter* genus. Former campylobacters have also been reassigned into *Arcobacter*, which contains the 'aerotolerant' campylobacters and *Sulfurospirillum*, which contains only free living environmental strains. Comparative genome analysis has also been carried out on the 25 genomes, of various levels of completeness, which are available for the *Campylobacteraceae* and related bacteria, including *Helicobacter*. The species tree generated using this method compares well with the phylogenetic tree derived from 16S rRNA gene sequences (Debruyne *et al.*, 2008).

Currently the genus *Campylobacter* contains 17 species and six subspecies, together with several named biovars which have clinical or economic importance (Debruyne *et al.*, 2008; On, 2005); *Campylobacter fetus* is the type species of the genus. Two species, *C. jejuni* and *C. coli*, are in particular the most likely to cause human *Campylobacter* infections. *C. jejuni* is believed to account for approximately 90% of all cases of human *Campylobacter* infections, with *C. coli*, which is usually associated with pigs, accounting for 3-4% of cases (Frost *et al.*, 1999).

## 1.3 *Campylobacter jejuni*

### 1.3.1 Morphology

*Campylobacter jejuni* cells are slim (0.2 - 0.8 µm wide and 0.5 - 5 µm long), spiral shaped, Gram-negative, non-sporulating rods that exhibit a characteristic rapid 'darting' motility, by means of a single unsheathed polar flagellum at one or both ends of the cell. The spiral morphology is useful for identification in a clinical context, as few bacteria of any clinical importance are spiral in shape; the morphology of *C. jejuni* is probably an adaptation to aid motility in viscous environments, such as that of the intestinal mucosa.

Having entered stationary phase or following exposure to suboptimal conditions or stressors *C. jejuni* has been proposed to enter a viable but nonculturable state (VBNC; Moore, 2001; Pearson *et al.*, 1993). This state is characterised by the alteration of the cellular morphology from the usual spiral shaped rod to a coccoid form, alterations in cell wall structure leading to changes in Gram staining (Moran and Upton, 1986; Ng *et al.*, 1985) and a decrease in metabolic activity. Whether campylobacters can reverse those changes which occur upon entry into the VBNC state, and so use this state as a means of avoiding stress, is not entirely clear. Some studies report that cells in a VBNC state are unable to revert to a form which is culturable and able to colonise (Hald *et al.*, 2001; Ziprin *et al.*, 2003). Others report that passage through the intestines of chicks (Talibart *et al.*, 2000) or mice (Baffone *et al.*, 2006) is sufficient to produce viable cells capable of colonisation. The ability of campylobacters to survive stress by entering a VBNC state, if this is in fact the case, could help to explain the prevalence and routes of transmission of this pathogen.

### 1.3.2 Growth requirements

*Campylobacter* spp. are fastidious, strictly microaerophilic organisms, requiring an O<sub>2</sub> concentration of 3 - 15% and a CO<sub>2</sub> concentration of 3 - 5% (Ketley, 1997); as such an atmosphere of 5% O<sub>2</sub>, 10% CO<sub>2</sub> and 85% N<sub>2</sub> is commonly used when culturing campylobacters. *C. jejuni* is thermophilic and has an optimum growth temperature of 42°C, a characteristic that may reflect an adaptation to the temperatures found in the intestines of mammals and particularly birds, which are usual habitats of *Campylobacter* spp; however a temperature of 37°C is commonly used when culturing pathogenic *Campylobacter* species. *C. jejuni* has a respiratory metabolism and is not able to ferment or oxidise carbohydrates, instead it obtains energy from amino acids and intermediates of the tricarboxylic acid cycle (Mohammed *et al.*, 2004). *C. jejuni* and *C. coli* both encode only one gene for catalase, *kata*, which catalyses the breakdown of H<sub>2</sub>O<sub>2</sub> to H<sub>2</sub>O and O<sub>2</sub> (Grant and Park, 1995). *C. jejuni* also has only a single superoxide dismutase (SOD) gene, which catalyses the breakdown of superoxide to H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub>; *E. coli* harbours multiple SOD genes (Cabiscol *et al.*, 2000). Despite the presence of these enzymes campylobacters are generally highly sensitive to both free radicals and superoxides.

### 1.3.3 Epidemiology

*Campylobacter* enteritis is the most common form of acute bacterial enteritis in the developed world; it affects people of all ages, but most particularly children aged less than 4 years and adults aged 15-44 years (Tauxe, 2005). There was a steady increase in *Campylobacter* infections in England and Wales up to a peak of 58,059 reported cases in 1998. Since this peak cases have declined slowly, although with significant variation in the number of cases in a given year, the general decreasing trend is believed to be due to better

control of infection and contamination in animals, particularly in poultry (Tam, 2001). In England and Wales during 2008 there were 49,800 laboratory confirmed cases of campylobacteriosis (Anon, 2009). It is believed that the true number of cases of *Campylobacter* infections is as much as 10 times greater than that recorded due to under-reporting, as many of those suffering from food-borne campylobacteriosis do not seek medical assistance. In temperate climates infection is seasonal, with twice the number of infections occurring in summer than in winter (Anon, 2006). The natural reservoir of *Campylobacter* spp. consists of both wild and domestic animals, with poultry having the greatest potential for causing human infection.

Campylobacteriosis is usually a food-borne infection and foods of animal origin play an important role in infection; raw and inadequately treated milk and untreated water have also been implicated as sources of infection (Frost *et al.*, 2002). However, food-borne outbreaks from a common source are unusual, and sporadic (i.e. single individual) food-borne infections make up the majority of cases. Despite the presence of large numbers of motile organisms in the faeces of infected persons and an apparently low infectious dose of between 500 and 800 organisms (Black *et al.*, 1988; Robinson, 1981), person-to-person transmission is rare. Many cases of campylobacteriosis are linked to foreign travel and the consumption of contaminated food or water in the areas visited.

In developing countries campylobacters are often hyper-endemic and *C. jejuni* infection is almost ubiquitous in children under 2 years old; there is often significant mortality associated with *C. jejuni* in these populations. *Campylobacter* enteritis is rarely seen in adults in areas where campylobacters are hyper-endemic as immunity is usually acquired early in life due to frequent exposure (Coker *et al.*, 2002).

### 1.3.4 Motility

*Campylobacter* is characterised by a rapid, darting motility mediated by bi-polar flagella. Lee *et al.* (1986) have also shown that the motility of *C. jejuni* increases with the viscosity of the solution, with a maximum reported speed of  $75\mu\text{m.s}^{-1}$  under highly viscous conditions. This ability to move quickly in viscous conditions could facilitate movement into and through the mucus covered and hence highly viscous lining of the intestinal tract.

Structurally the flagellum consists first of the basal body, which connects the flagellum to the cell and also interacts with the flagellar motor. The hook links the basal body to the flagellar filament, which is composed of thousands of copies of two proteins, FlaA and FlaB. The genes encoding FlaA and FlaB are adjacent to one-another and share >93% sequence homology, and are independently transcribed, *flaA* from a  $\sigma^{28}$  promoter and *flaB* from a  $\sigma^{54}$  promoter (Guerry *et al.*, 1990). Mutants in *flaA* have been shown to be poorly motile and to possess only short, truncated flagella; mutants in *flaB* however possessed near-normal length flagella and were almost as motile as the parent strain but did show some reduction in pathogenic potential (Guerry *et al.*, 1991; Wassenaar *et al.*, 1993). This would appear to suggest that FlaA is the major flagellin, whilst FlaB forms a minor part of the flagellar structure and is possibly not expressed under all conditions. Whether this is in fact the case is uncertain as is the exact location or function of FlaB in the flagellar filament. The presence of two homologous *fla* genes may confer an advantage in the event of a mutation occurring in one copy, such that the organism can still maintain at least a certain level of motility, Wassenaar *et al.*, (1991).

The flagellin from *C. jejuni* 81-176 is predicted from its amino-acid sequence to be 59.5kDa in size, however flagellin from this strain is actually approximately 65KDa in apparent (i.e.  $M_r$ ) size. The additional 10% mass consists of glycosyl groups which are

attached by an *O*-linkage to 19 serine and threonine residues in the central, surface exposed region of the flagellin of strain 81-176 (Thibault *et al.*, 2001). At least some glycosylation of flagellin is required in order to form a functional flagellar filament (Linton *et al.*, 2000); although the exact function(s) of flagellin glycosylation is as yet unclear, flagellin glycosylation is covered in greater detail in Section 1.8.

### 1.3.5 Biofilm formation

As previously stated *C. jejuni* is one of the leading causes of bacterial gastroenteritis worldwide and as such it is very widespread. Paradoxically its microaerobic growth requirements, together with its inability to tolerate atmospheric oxygen concentrations, mean that it is unable to multiply in the aerobic environment. Transmission of *C. jejuni* between hosts, particularly livestock hosts, is likely to involve a period of exposure of *Campylobacter* to the hostile external environment; the methods used by *Campylobacter* to survive in the aerobic environment are not well understood. It has been suggested by Buswell *et al.* (1998) that *C. jejuni* is able to maintain itself in the environment by forming a biofilm.

Biofilms are usually defined as multi layered bacterial populations enclosed in a matrix composed of extracellular polysaccharide substances; these bacteria may be adherent to each other and to surfaces or interfaces (Costerton *et al.*, 1995). Aggregations of bacteria attached to each other, but not to a surface, are usually termed ‘flocs’ and have many properties in common with surface attached biofilms (Hall-Stoodley *et al.*, 2004); aggregates of bacterial cells that form at an air-liquid interface are termed ‘pellicles’ (Friedman and Kolter, 2004). Biofilms exist in a wide variety of ecological niches and are of considerable economic importance in the food industry, waste water treatment plants and in medicine. Monospecies biofilms are formed by a number of human pathogens, including

*Staphylococcus epidermidis* (Mack *et al.*, 2000) *Vibrio cholerae* (Nesper *et al.*, 2001) and *Pseudomonas aeruginosa*, the latter in the lungs of cystic fibrosis patients (Whiteley *et al.*, 2001). Bacteria in biofilms are often less susceptible to antibiotics than their free-living counterparts (Nichols, 1991); protection against other stresses, including desiccation, nutrient starvation and the host immune system, may also be conferred by biofilms (Davies, 2003; Miller and Bassler, 2001), allowing bacteria to survive what would otherwise be lethal environments.

Whilst there have been no reports of *C. jejuni* biofilm formation *in vivo* this strain has been found in biofilms with several other bacterial species (Trachoo *et al.*, 2002). There is considerable variation in the ability of strains of *C. jejuni* to form mono-species biofilms. Several strains, including NCTC 11168 and 81-176 have been shown to form biofilms on a variety of surfaces including stainless steel, glass, poly-vinyl chloride and nitrocellulose membranes (Kalmokoff *et al.*, 2006), although some clinical isolates seem to be impaired in biofilm formation (Joshua *et al.*, 2006). *C. jejuni* has been shown to form the three distinct forms of biofilm when in monoculture; it can form a true biofilm by attaching to a glass surface, a pellicle at an air-liquid interface when grown in stationary liquid culture, and unattached cellular aggregates, or flocs, in the culture medium (Joshua *et al.*, 2006). It was also demonstrated that, unlike free-living cells, the bacteria in the attached biofilm and pellicle retained their characteristic spiral morphology and were viable under atmospheric oxygen conditions for several weeks.

Kalmokoff *et al.* (2006) demonstrated that during growth in a biofilm, *C. jejuni* cells express genes involved in energy generation, such as succinyl-CoA synthase, and in biosynthesis (riboflavin synthase) at higher levels than their free-living counterparts; these expression differences are commensurate with those seen in other bacteria (Hefford *et al.*,



2005; Lazazzera, 2005). However, a particular group of proteins present at higher levels in the biofilm inhabiting *C. jejuni* cells, the motility related proteins, were unusual and are not normally identified as up-regulated in other biofilm-grown bacteria (Kalmokoff *et al.*, 2006). The motility related proteins with enhanced expression included the flagellin structural proteins, hook, basal body proteins, filament cap and the chemotaxis protein CheA. It is known that flagella are important in the initial attachment phase of biofilm formation (Kirov *et al.*, 2004), but in some genera motility genes are commonly repressed after the attachment phase has been successfully completed (Lazazzera, 2005). This makes the enhanced expression of motility-related proteins in *C. jejuni* inhabiting mature biofilms unusual and thus far inexplicable. It was confirmed by Reeser *et al.* (2007) that an aflagellate *AflaAB* mutant in *C. jejuni* M129 was deficient in its ability to form biofilms. Joshua *et al.* (2006) also reported that an aflagellate *maf5* mutant (now annotated *pseE*; Cj1337), which appears to have an as yet unclear role in the assembly and transfer of glycans to flagellin monomers (McNally *et al.*, 2006a), and *fliS* mutants in *C. jejuni* NCTC 11168 did not attach to surfaces and so were unable to form biofilms or pellicles; these strains were however able to form flocs in the body of the media.

### 1.3.6 The Nitrosative stress response in *C. jejuni*

At elevated levels nitric oxide (NO) reacts easily with a variety of targets within a bacterial cell including thiols and metal centres, such as haem centres and Fe-S clusters (Poole and Hughes, 2000); therefore NO can have a strong bactericidal activity. NO from a variety of sources can be encountered by bacteria, both in the environment, where NO is present as an intermediate of denitrification, and also in the mammalian host, where NO is present due to the reaction of salivary nitrite with stomach acid (Lundberg *et al.*, 2004) and as a prominent

agent in macrophage killing. The primary source of NO in the mammalian host are nitric oxide synthases, particularly inducible NO synthase 2, which is expressed primarily in macrophages after induction by cytokines or bacterial products, including lipooligosaccharides (Ding *et al.*, 1988; Iovine *et al.*, 2008). Both systemic (*C. jejuni* 84-25) and diarrhoeal (*C. jejuni* 81-176) *Campylobacter* were shown by Iovine *et al.* (2008) to be killed by cytokine or LOS stimulated macrophages in an inducible NO synthase dependant manner. NO is lipophilic and able to diffuse freely, so it is able to enter cells without restriction. Bacteria have a variety of responses to NO exposure, most of which are designed to detoxify NO and its associated redox products (Poole, 2005) and can modulate expression of these responses according to NO exposure.

*C. jejuni* is thought to encounter increased levels of NO during the course of a human infection, as the synthesis of NO in patients with infective bacterial gastroenteritis is significantly increased (Forte *et al.*, 1999), including following an infection with *Campylobacter* (Enocksson *et al.*, 2004). Elvers *et al.* (2004) demonstrated that the single domain globin Cgb performs the major NO scavenging and detoxification function in *C. jejuni*. Expression of *cgb* is minimal in laboratory media but is upregulated strongly and specifically upon exposure to nitrosative stress. The NO-sensing regulator of *cgb* expression in *C. jejuni* is NssR (nitrosative stress-response regulator) encoded by Cj0466, which is the only member of the Crp-Fnr transcriptional regulator superfamily present in *C. jejuni* (Elvers *et al.*, 2005; Parkhill *et al.*, 2000); the mechanism by which NssR senses NO is unclear. NssR regulates the expression of a small nitrosative stress response regulon of at least four genes, which include *cgb* and *ctb*, a truncated globin the physiological function of which is unclear, and two genes of unknown function, Cj0761 and Cj0830. The genes *cgb* and *ctb* also appear to be positively regulated by oxygen, which may indicate a role for oxygen in either NO

detoxification or NssR activity (Monk *et al.*, 2008). The study by Monk *et al.* (2008) also indicated that the *Campylobacter* response to NO stress is more general than previously thought, as several heat shock associated proteins, including HrcA, GrpE and DnaK, were upregulated in response to NO stress, potentially these proteins are involved in the response to the accumulation of unfolded proteins following NO exposure. Also upregulated following NO stress were several proteins involved in iron uptake, as NO attacks proteins with haem centres or Fe-S clusters the increased uptake of iron may be required for restoration of normal protein function.

The promoter regions of those genes regulated by Crp-Fnr superfamily members usually contain distinctive recognition motifs, such as the inverted repeat sequence TTGAT-N<sub>4</sub>-ATCAA recognised by FNR family proteins (Eiglmeier *et al.*, 1989). Elvers *et al.* (2005) discovered a similar motif, which has the consensus sequence TTAAC-N<sub>4</sub>-GTAA, upstream of all of the NssR-dependent genes that they identified; this sequence is therefore predicted to be the NssR-binding motif under conditions of nitrosative stress; whether it is still used in conditions where nitrosative stress is not present is unknown.

## 1.4 Clinical manifestations of *C. jejuni* infection

*C. jejuni* can cause a broad spectrum of diseases ranging from gastroenteritis, septicaemia, meningitis and abortion, to autoimmune diseases such as reactive arthritis and Guillain-Barre syndrome. However, the most common result of human infection with *C. jejuni* is an acute, self-limiting gastrointestinal illness; symptoms usually include diarrhoea, fever and abdominal cramps, although the variation of symptoms between individuals is common (Young and Mansfield, 2005). The incubation period after ingestion of *C. jejuni* varies from one to five days (Allos 2001), although this can be dependant on the number of

cells ingested. The symptoms are often indistinguishable from other acute bacterial gastrointestinal infections such as salmonellosis and shigellosis and a definitive diagnosis is only made after detecting *C. jejuni* in the faeces.

The clinical manifestations of *C. jejuni* infection vary between developed and developing countries. In developed countries primary infection usually results in inflammatory diarrhoea, which is characterised by prominent fever, abdominal pain and stools containing leukocytes, mucus and gross or microscopic blood. This form of gastroenteritis tends to be self-limiting and is usually resolved within a week (Black *et al.*, 1988). In developing countries a spectrum of disease exists, ranging from severe inflammatory illness to mild non-inflammatory diarrhoea and an asymptomatic carrier state (Young and Mansfield, 2005). Infants and young children tend to be the only groups that have symptomatic disease and within these groups non-inflammatory, watery diarrhoea is most common (Blaser *et al.*, 1980). In these circumstances adults rarely have symptomatic disease and it is in these groups that the asymptomatic shedding of *C. jejuni* has been observed (Pazzaglia *et al.*, 1991).

#### **1.4.1 Complications arising from *C. jejuni* infection.**

The majority of *Campylobacter* infections resolve naturally, without the need for antibiotic treatment, but complications tend to arise if the organism spreads from the gastrointestinal (GI) tract. Local infections, within the GI tract area, such as appendicitis, cholecystitis and pancreatitis can occur together with gastrointestinal haemorrhaging (Allos, 2001). More rare are extra-intestinal infections such as meningitis, endocarditis, septic arthritis, osteomyelitis and neonatal sepsis. Bacteraemia is also rare, reported in less than 1% of cases of *Campylobacter* infection (Allos, 2001) and these patients are likely to be at the extremes of age or have an underlying immune compromise. There are also several late-onset

complications following *Campylobacter* infection which are auto-immune in nature including, reactive arthritis, Miller Fisher syndrome, and Guillain-Barre syndrome. Recently irritable bowel syndrome (IBS), characterised by abdominal pain and altered bowel habit, has been associated as a post-infectious manifestation, with enteric *C. jejuni* infection (Spiller, 2007). Thornley *et al.* (2001) demonstrated that IBS was occurring in individuals who had suffered *Campylobacter* enteritis at a greater rate than expected, given the control data. This study also found that *C. jejuni* isolates that were toxic to Hep-2 cells were more frequently isolated from those individuals who went on to develop IBS.

#### 1.4.1.1 Guillain-Barre syndrome (GBS)

GBS is the most common and most serious sequela of infection of *C. jejuni* and, since the eradication of polio in most areas of the world, has become the most common cause of acute neuromuscular paralysis (Nachamkin *et al.*, 1998). GBS is an autoimmune disorder of the peripheral nervous system and is characterised by muscle weakness and abnormal sensations, including tingling, numbness and vibrations, which are usually symmetrical and evolve over the course of several days, usually beginning 1-3 weeks after the onset of diarrhoeal illness. Severity varies between patients; in the most severe cases facial and eye movements become abnormal and breathing muscles are weakened, leading to the need for pulmonary support. The disease is self-limiting and many patients make a complete recovery, although 15-20% of GBS patients are left with severe neurological defects (Nachamkin *et al.*, 1998). It is estimated that the incidence of GBS is less than one case per 1000 *C. jejuni* infections and that *C. jejuni* is the trigger for 30% of cases of GBS (Allos, 1997). The pathogenesis of post-*Campylobacter* GBS is believed to involve molecular mimicry, whereby patients who suffer from GBS following *C. jejuni* infection develop antibodies to the bacterial

lipooligosaccharides (LOS), specifically to the LOS outer core, and these antibodies cross-react with peripheral nerve cell surface gangliosides (Young and Mansfield, 2005). This would then cause the host immune system to attack the myelin sheath on host neurons. This is supported by the observation that the acute stage of the disease usually coincides with the highest serum titres of antiganglioside antibodies. This molecular mimicry is thought to involve the presence of sialic acid in the LOS; it has been observed that specific combinations of genes are required to allow *C. jejuni* to express ganglioside mimics (Bersudsky *et al.*; 2000, Godschalk *et al.*, 2007).

#### 1.4.1.2 Miller Fisher Syndrome (MFS)

MFS is a variant of GBS which is characterised by ataxia (loss of coordination), ophthalmoparesis (paralysis of the eye muscles) and areflexia (loss of tendon reflexes). The development of the disease is more gradual than that of GBS and a complete recovery is usually made over weeks or months. The cause of MFS is believed to be a molecular mimicry similar to that of GBS and in cases of MFS, serum antibodies that recognise the gangliosides GQ1b are often identified (Ang *et al.*, 2002).

#### 1.4.1.3 Reactive arthritis (ReA)

ReA is a non-purulent joint inflammation which can be triggered by infections in the gut or urogenital tract. Apart from *C. jejuni*, other bacteria known to trigger ReA include *Salmonella*, *Yersinia enterocolitica* and *Shigella flexneri* (Hannu *et al.*, 2002). The main features of ReA are inflamed joints, inflammation of the eyes and inflammations of the urogenital or gastrointestinal systems, without apparent local infection. *C. jejuni* antigens have been shown to be present in affected areas, but no viable organisms have been detected

(Smith, 1996). The inflammation is believed to result from the response of synovial T-cells to any bacterial antigens present. ReA may occur following 0.7- 7% of cases of *Campylobacter* enteritis (Bremell *et al.*, 1991; Hannu *et al.*, 2002).

#### 1.4.2 Treatment of *C. jejuni* infection

Generally, *Campylobacter* enteritis is a self-limiting disease and supportive measures such as fluid and electrolyte replacement are the only therapies necessary for the majority of patients. Antibiotic therapy is indicated for patients who are acutely ill with enteritis or are HIV positive or otherwise immunocompromised (Butzler, 2004). Macrolide antibiotics, such as erythromycin, clarithromycin and azithromycin are the drugs most commonly used to treat *C. jejuni* infection, due to their ease of administration, lack of serious toxicity, low cost, high efficacy and narrow spectrum of activity (Allos, 2001). Fluoroquinolone antibiotics, such as ciprofloxacin, may also be used, with the advantage that other bacterial pathogens capable of causing gastroenteritis are also susceptible, removing the need to wait for laboratory test results. However, an increasing number of strains worldwide have been found to be developing resistance to fluoroquinolone antibiotics, possibly due to the use of substances related to these drugs in poultry farming (Snelling *et al.*, 2005).

Vaccination is a possible option for the control of *Campylobacter*. Immunity against campylobacters has been demonstrated in several groups including: those individuals previously infected (Black *et al.*, 1988), in breast-fed infants whose mothers have been recently exposed (Nachamkin *et al.*, 1994) and in abattoir workers (Cawthraw *et al.*, 2000). Orally administered vaccines have been most frequently evaluated, as the vaccine would need to stimulate intestinal immunity to be effective against *Campylobacter*. Baqar *et al.* (1995) demonstrated that an inactivated whole-cell vaccine produced immunity in primates and Lee

*et al* (1999) used a whole-cell vaccine plus purified flagellin to produce some immunity in a mouse model. The *C. jejuni* capsule has also been investigated for its potential to deliver vaccine candidates (Baqar *et al.*, 2007).

## 1.5 Pathogenesis of *C. jejuni*

Whilst *C. jejuni* has been recognised as an important human pathogen for more than 30 years, detailed knowledge of its pathogenicity factors remains elusive. *C. jejuni* is a food-borne pathogen and as such enters the host via ingestion in contaminated food or water; inocula as low as 800 cells were found by Black *et al.* (1988) to cause disease in humans. Campylobacters enter the host intestine by passing through the stomach acid barrier and colonise the mucus layer covering the epithelia of the distal ileum first and then that of the colon (Ketley 1997); it is after colonisation of the colon that disease symptoms begin to appear (Black *et al.*, 1988). Having successfully colonised the mucus layer, campylobacters interfere with the absorptive capacity of the intestine by damaging epithelial cells by invasion and toxin production, resulting in inflammation and diarrhoea (Park, 2002). A series of processes are necessary for *Campylobacter* virulence; these include: 1) adhesion, 2) invasion, 3) toxin production and 4) intracellular survival and intestinal translocation; motility and chemotaxis are also necessary for virulence, especially in the early stages of infection.

### 1.5.1 Adhesion

Adhesion is thought to be fundamental to *C. jejuni* pathogenesis. Adhesins are molecules present on the surface of a bacterial cell that facilitate the attachment of the bacterial cell to host cell receptor molecules; this process is thought often to be essential for disease to occur. *C. jejuni* adhesins have been shown to be synthesised constitutively, as metabolically inactive (heat-killed and sodium-azide killed) *C. jejuni* were found to bind to



cultured cells at levels comparable with metabolically active organisms (Konkel *et al.*, 1992). A variety of outer-membrane proteins have been implicated in adhesion, as have other cell surface structures including lipooligosaccharide and the major outer membrane protein.

#### 1.5.1.1 Outer membrane proteins (Peb1, CadF and JlpA).

Peb1 was first identified by de Melo *et al.* (1990). It is a 28 kDa protein, homologous with membrane proteins involved in amino acid transport from other Gram-negative bacteria and has been shown by Pei *et al.* (1998) to mediate *C. jejuni* binding to epithelial cells. Konkel *et al.* (1997) characterised another outer membrane protein that facilitates *C. jejuni* binding to the host attachment protein fibronectin, an adhesive protein that provides structural support in epithelial cells. This 37 kDa protein was designated CadF and is also believed to enhance internalisation of *C. jejuni* into host cells. JlpA is a 42 kDa lipoprotein originally identified by Jin *et al.* (2001), and is believed to facilitate attachment of *C. jejuni* to epithelial cells but to play no role in internalisation.

#### 1.5.1.2 Lipooligosaccharides (LOS)

LOS and polysaccharide capsules are major surface antigens of *Campylobacter* and have been shown to have a role in virulence in a variety of pathogenic enteric bacteria, including a role in host cell binding and in some cases internalization in *E. coli*, *Salmonella typhi* and *Neisseria gonorrhoeae* (Konkel *et al.*, 2001). It has been suggested that structural changes in LOS could alter the organisms' surface charge, so affecting its binding potential; McSweeney and Walker, (1986) proposed that the binding of *C. jejuni* to cultured epithelial cells was mediated by LOS.

### 1.5.1.3 Major outer membrane protein (MOMP)

In 1997 Schröder and Moser proposed that the MOMP of *C. jejuni*, named PorA, an importance surface antigen which functions as a porin to facilitate the transfer of hydrophilic molecules across the outer membrane, could also serve as an adhesin, as it was found to bind to cultured cell membranes. The specificity and mechanism of MOMP binding to cultured cells has not been determined.

### **1.5.2 Invasion and protein secretion**

The symptoms of campylobacteriosis, including inflammation, the presence of blood and leukocytes in faeces and occasional bacteraemia, strongly suggest that invasion is an important pathogenic mechanism in *C. jejuni* as all of the above are products of the mammalian inflammation process; however, little is known about the exact mechanisms used to achieve this. Invasion of *C. jejuni* is strain dependent, with clinical isolates being more invasive than environmental strains (Konkel and Joens, 1989); the ability of cells to invade can be lost during extensive passage *in vitro* (Konkel *et al.*, 1990). *C. jejuni* does not constitutively synthesise proteins to induce uptake, as metabolically inactive (sodium-azide killed) cells are not internalised. Also chloramphenicol, a specific inhibitor of bacterial protein synthesis, prevents the internalisation of *C. jejuni* by host cells (Raphael *et al.*, 2005). However, blocking host cell protein synthesis with cyclohexamide does not inhibit *C. jejuni* invasion, suggesting that the host cell proteins required for invasion are present during normal cell growth (Oelschlaeger *et al.*, 1993).

Motility has been demonstrated by several studies as being necessary for invasion, although there is no evidence to support a direct role for any of the flagellar apparatus proteins in invasion by *C. jejuni* (Wassenaar *et al.*, 1991; Golden and Acheson, 2002; Carrillo

*et al.*, 2004). A range of bacterial proteins have also been linked to invasion as, following co-cultivation of *C. jejuni* with INT407 cells, *C. jejuni* was seen to produce at least 14 additional proteins, in comparison to bacteria cultured without cells (Konkel and Cieplak, 1992). Later studies by Konkel *et al.* (1993) identified nine new proteins relative to media-grown bacteria, using rabbit antisera; one of these proteins, CiaB (Campylobacter invasion antigen B), has been found to be necessary for the secretion of the other eight (Konkel *et al.*, 1999). CiaB has also been shown, by immunofluorescence microscopy, to translocate into infected host cells during the infection process. Rivera-Amill *et al.* (1999) proposed that its translocation occurred via a type III-like secretion process, as the Cia proteins conform to the criteria of type III secretion proteins. The only type III-like secretion apparatus encoded by *C. jejuni* NCTC 11168 is the flagellar apparatus (Parkhill *et al.*, 2000), Larson *et al.* (2008) demonstrated that CiaB could hypothetically be secreted via the *C. jejuni* flagellar type III-like secretion system. Poly *et al.* (2007) showed that a minimal flagellar structure was necessary for the secretion of the novel virulence factor FspA, which is not thought to be involved in adherence or invasion; one version, FspA2, has been shown to be associated with host cell monolayers during induced apoptosis. The secretion of proteins via the flagella apparatus has also been reported in other bacteria including *Yersinia enterocolitica* (Young *et al.*, 1999) and *Bacillus thuringiensis* (Ghelardi *et al.*, 2002) and Konkel *et al.* (2004) found that mutations that affected either the export of flagella components or the non-filament structural components of *C. jejuni* 81-176 also resulted in a secretion-negative phenotype.

### 1.5.3 Toxins

Campylobacters reportedly produce a variety of toxic activities, but most current work is centred on an exotoxin that is a member of the cytolethal distending toxin (CDT) family

and is the only fully defined toxin encoded in the genome sequence of *C. jejuni* NCTC 11168 (Parkhill *et al.*, 2000). CDT was first discovered in *E. coli* and also known to be produced by certain *Shigella* and *Salmonella enterica* strains and is encoded by many *C. jejuni* strains (Johnson and Lior, 1988; Lara-Tejero and Galan, 2000). It is encoded by three adjacent genes *cdtA*, *cdtB* and *cdtC*; the holotoxin consists of one copy of each gene product (Lara-Tejero and Galan, 2001; Pickett and Lee, 2005). CDT appears to be toxic to a wide variety of cultured mammalian cell lines, apparently by blocking cell division by arresting the cell cycle at the G2/M phase, after chromosome duplication but before chromosome separation and cell division. Elwell and Dreyfus, (2000) have shown that the CdtB subunit of the toxin has homology to certain mammalian DNases and is responsible for bringing about cell cycle arrest by degrading the host cell chromosomes, but it is not clear what role the CDT has in human infection *in vivo*.

### **1.5.4 Intracellular survival and intestinal translocation**

#### **1.5.4.1 Intracellular survival**

Having colonised the mucus layer of the intestines, campylobacters invade intestinal epithelial cells and interfere with the absorptive capacity of the intestine, resulting in inflammation and diarrhoea (Park, 2002). There appear to be three key stages in *C. jejuni* interactions with nonphagocytic cells that contribute to this, mediation of uptake into cells, avoidance of lysosomes by the modification of vesicular trafficking and stimulation of the release of proinflammatory cytokines. Several studies have investigated the fate of campylobacters after entering host cells, in general it has been found that *C. jejuni* cells lose viability over a 24 hour period when within intestinal epithelial cells (Candon *et al.*, 2007; Day *et al.*, 2000). However, Watson and Galan (2008a) demonstrated, using LIVE/DEAD

staining, that there is no significant decrease in *C. jejuni* viability after 24 hours within epithelial cells, but that these cells could no longer be cultured using standard laboratory conditions and additional oxygen limiting conditions were necessary for the successful growth of these cultures. *C. jejuni* cells have been found localized in membrane-bound vacuoles, so called *C. jejuni* containing vacuoles (Kiehlbauch *et al.*, 1985; Russell and Blake, 1994). It is also apparent that *C. jejuni* has developed a method of altering normal host epithelial cell traffic such that these vacuoles are not delivered to lysosomes (Watson and Galan, 2008b). However, these alterations do not function in macrophages as *C. jejuni* containing vacuoles are targeted to lysosomes and hence destroyed in these cells (Kiehlbauch *et al.*, 1985; Wassenaar *et al.*, 1997). *C. jejuni* is susceptible to NO generated both by the reaction of dietary nitrate with gastric acid in the stomach and by the nitric oxide synthases of macrophages (Iovine *et al.*, 2008). Resistance to NO stress is regulated by NssR, the nitrosative stress response regulator and detoxification appears to be carried out largely by Cgb, a single domain globin; also regulated by NssR are at least four other genes of unknown functions (Elvers *et al.*, 2005). Also produced by host immune cells, particularly neutrophils and macrophages are reactive oxygen species, which can include superoxides and hydrogen peroxide. *C. jejuni* is sensitive to reactive oxygen species and encodes several genes to enhance its resistance, including *katA*, which encodes catalase to breakdown hydrogen peroxide to H<sub>2</sub>O and O<sub>2</sub> (Grant and Park, 1995). *C. jejuni* also encodes a single superoxide dismutase (SOD) gene, *sodB*, which catalyses the breakdown of superoxide to H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub> (Cabiscol *et al.*, 2000).

#### 1.5.4.2 Intestinal translocation

The ability of a pathogen to migrate across a cell barrier by invading cells can be an important virulence factor, as it allows access to underlying tissues and dissemination through a host organism. However, *C. jejuni* can also translocate across the epithelial cell barrier in an alternative invasion mechanism and occasionally may migrate from the intestinal mucosa to a variety of extra-intestinal sites, resulting in complications including cholecystitis and septicaemia. The mechanism of translocation, which involves the bacteria moving between host cells rather than being internalised by them, is unclear, although Bras (1999) reported that functional flagella and *de novo* protein synthesis is required. Grant *et al.* (1993) also reported that mutants in *flaA* were unable to cross epithelial cell monolayers, indicating that FlaA is somehow essential for translocation. Harvey *et al.* (1999) found no correlation between an isolate's invasive and translocation potential, indicating that the genes encoding the products necessary for invasion are different from those that confer translocation ability. This may also suggest that *C. jejuni* may translocate a cell monolayer using a paracellular route (between cells) rather than a transcellular route (through cells), as the ability to invade would not be necessary for the former type of movement (Raphael *et al.*, 2005).

#### **1.5.5 Chemotaxis and Motility**

Both motility and chemotaxis have been shown to have an important role in host colonisation and virulence (Yao *et al.*, 1997). Chemotaxis is necessary for the invasion of host tissues and as such campylobacters possess mechanisms to detect chemical gradients, which are linked to motility functions, enabling them to move up and down chemical gradients (Snelling *et al.*, 2005). The genome sequence of *C. jejuni* NCTC 11168 revealed the presence of orthologues of several genes involved in chemotaxis, the *che* genes, and aerotaxis, *aer*

genes, in *E. coli* and other bacteria; also present are 10 chemoreceptor and two aerotaxis receptor orthologues (Parkhill *et al.*, 2000; Korolik and Ketley, 2005). Chemo-attractants of *C. jejuni* include mucin components, including L-fucose and L-serine and particular organic acids, such as pyruvate and succinate (Hugdahl *et al.*, 1988).

Chemotaxis is possible only if the organism is sufficiently motile to move along the detected gradient; *C. jejuni* is highly motile by means of a single, unsheathed flagellum at one or both ends of the cell, allowing the organism to travel at up to  $75\mu\text{m.s}^{-1}$  (Carrillo *et al.*, 2004). The combination of the flagellum and the spiral cell shape are believed to give campylobacters high motility in viscous environments, such as the mucus layer in intestines. Whilst it appears that flagella do not have a role in adherence, as non-flagellated *C. jejuni* cells were able to adhere to epithelial cells as effectively as wild-type cells (Grant *et al.*, 1993; Nachamkin *et al.*, 1993), it does appear that flagella have a role in the internalisation process. Aflagellate mutants were internalised into host cells at a considerably lower level than the wild-type (Grant *et al.*, 1993). It has been hypothesised by Yao *et al.* (1994) that the flagellum may also be able to act as a type III-like secretion mechanism, for the secretion of virulence proteins; *Campylobacter* lacks an independent type III system.

## 1.6 The genome of *C. jejuni* NCTC 11168

### 1.6.1 The genome sequencing project

The genome sequence of *C. jejuni* NCTC 11168 was completed in 2000 by Parkhill *et al* and was the first *Campylobacter* genome to be sequenced. The genome comprises a circular chromosome 1,641,481 base pairs in size, with a G+C content of 30.6% which is predicted to encode 1,654 proteins and 54 stable RNA species. It is one of the densest bacterial genomes sequenced, with about 94.3% of the genome thought to encode proteins.

The genome has several remarkable features, one of which was that it was almost devoid of phage-associated sequences and contained few repeat sequences, only four within the complete genome. Another important feature is the presence of homopolymeric runs of ca. 9-13 nucleotides, known as hypervariable sequences, which were often seen in genes responsible for the biosynthesis and modification of surface structures or in other genes of unknown functions closely associated to these genes. These hypervariable sequences would, by slipped strand mis-pairing, allow expression of these genes to be phase variable, producing a high frequency of on and off changes and which may explain the capacity of *C. jejuni* to produce phase variation in the carbohydrate moieties present in surface structures such as flagella. The size of the chromosome means that the number of genes is limited, approximately 1,600 in *C. jejuni* NCTC 11168 as opposed to more than 5,000 in *Salmonella*, which may help to explain why *Campylobacter* has comparatively fastidious growth requirements. When the flagellar genes were compared to those of the *E. coli*/*Salmonella* model flagellar system, some genes were absent, including several regulatory genes such as the *flhDC* master regulator system, while others were present in addition. All gluconeogenesis genes and those of the tricarboxylic acid pathway were present, but at least one of the genes of the glycolytic pathway was lacking. About 55.4% of the genes have orthologues in *H. pylori*, many of which have housekeeping functions.

Following the sequencing of this first genome by Parkhill *et al.* (2000) several other *C. jejuni* sequences have been completed including *C. jejuni* 81-176 (Hofreuter *et al.*, 2006) and *C. jejuni* RM1221 (Fouts *et al.*, 2005). Certain non-*jejuni* *Campylobacter* have also been sequenced including *C. coli* RM2228, a multidrug-resistant poultry isolate (Miller, 2008) and a human clinical isolate of *C. lari*, RM2100 (Miller, 2008). The availability of multiple *Campylobacter* genome sequences allows the application of comparative genomics



approaches to identify virulence determinants and metabolic pathways common to the majority of campylobacters, as well as the identification of genes and/or pathways specific to particular *Campylobacter* species, which could potentially be linked to differences host-range, colonisation and virulence which are already known. With regards to flagellin glycosylation, the availability of multiple genome sequences has allowed the diversity of flagellin glycosylation associated genes, both in terms of number and type, to be identified.

### **1.6.2 The sequenced NCTC 11168 strain differs from the original isolate.**

Following the completion of the genome sequence of *C. jejuni*, it was observed that the genome sequenced *C. jejuni* NCTC 11168 strain was flagellate but non-motile, and defective in its ability to colonise both one-day old chicks (Ahmed *et al.*, 2002) and SCID mice (Hodgson *et al.*, 1998). These differences were believed to have arisen due to extended subculturing of the 11168-GS WT strain over a period of years (Gaynor *et al.*, 2004). The strain was first isolated by Skirrow in 1977 from human diarrhoeic faeces and was subsequently added to the National Collection of Type Cultures (NCTC) where the strain was subcultured to provide both a freeze-dried stock and for distribution to other laboratories; it was from this subcultured background that the genome-sequenced strain was derived, which here is designated 11168-GS WT. However, when the 11168-GS WT strain was originally isolated, frozen stocks were also kept separately to that accessed to the NCTC. These stocks are thought not to have been subcultured so extensively and were presented to the Veterinary Laboratories Agency in 1999; it is from this background that the *C. jejuni* 11168 ‘original’ strain is derived, which here is designated 11168-O WT. Gaynor *et al.* (2004) found that the original strain, unlike the genome-sequenced strain, was able to colonise one-day old chicks extremely well. In the same study, differences in morphology were also observed; the original

strain was spirally shaped as is expected in *Campylobacter*, whereas the genome-sequenced strain had a straight, rod-like morphology. Whilst both isolates retained polar flagella, the genome-sequenced strain was deficient in motility in both broth and soft agar, in contrast the original strain was highly motile on both substrates and in broth showed the fast, darting motility characteristic of *Campylobacter*.

Whilst no significant difference in the DNA sequence of the two isolates was found, microarray experiments showed that several clusters of genes were differently expressed in the genome-sequenced and original strains. Several respiratory and metabolic genes, including genes involved in the TCA cycle and in gluconeogenesis, were expressed at higher levels in the original strain compared to genome-sequenced, under both microaerobic and severely oxygen-limited conditions. A number of flagellar genes were also expressed at significantly higher levels in the original strain, particularly under severely oxygen-limited conditions; these genes included several putative hook and basal body structures. The major flagellins *flaA* and *flaB* were expressed at comparable levels in both variants, together with other known flagellar genes. Gaynor *et al.* (2004) hypothesised that these expression differences were due to the fact that the original strain was better adapted to the oxygen-limited conditions that would be encountered in the anaerobic parts of the gut. The genome-sequenced strain had become, over time, more adapted to the laboratory environment where it would be more likely to encounter atmospheric oxygen concentrations than the oxygen starvation experienced in the gut, hence it no longer responded to the stimulus of oxygen-limitation. The 11168-O WT used here may not be identical to that examined by Gaynor and co-workers in 2004, but its phenotype appears closely related.

The difference in motility between the two strains has also been examined by Turner and Penn (2005), who compared the whole cell protein profiles of the strains by SDS-PAGE.

The flagellin of the 11168-GS strain was seen to have a lower molecular mass than that of the 11168-O strain; a possible explanation for this is a variation in the post-translational modifications of the flagellin protein. *Campylobacter* flagellin has been shown by Thibault *et al.* (2001) to be one of the most extensively modified prokaryotic proteins known, with approximately 10% of the mass of the protein being made up of *O*-linked carbohydrates; a phenomenon which will be discussed in detail later.

## 1.7 Bacterial flagella

The flagellum is a structure which mediates bacterial motility, can act as a protein export apparatus and is also an important bacterial virulence factor. Flagellar biosynthesis has been most thoroughly studied in *Escherichia coli* K-12 and *Salmonella enterica* serovar Typhimurium LT2, as historically these bacteria were among the first studied in detail and are now used as model systems for the elucidation of processes involved in flagella assembly and function. A general model of bacterial flagellar structure, function, assembly and regulation has been derived from these studies. However, it is clear that there are many bacterial species, only distantly related to those used to form the model, that are flagellate and whose flagellar differ accordingly (Pallen *et al.*, 2005); *Campylobacter* should be considered one of these species.

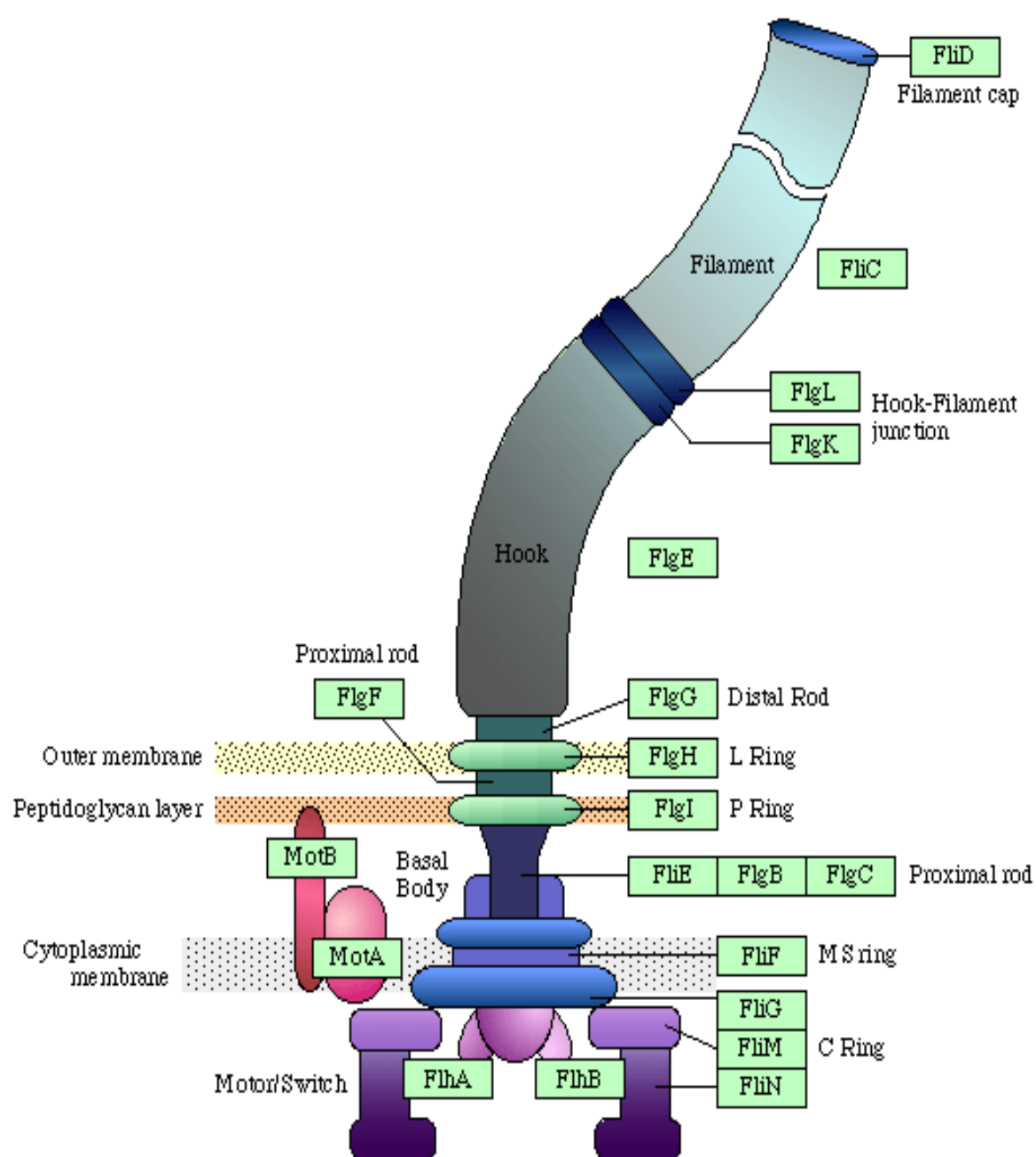
### 1.7.1 Flagellar structure

Bacterial flagella are long, spiral shaped hair-like structures that protrude from the surface of the cell. To produce thrust the flagellum is rotated by a membrane bound molecular motor located at its base. A flagellum consists of three structural subunits, a long helical filament, a short curved hook structure and the basal body, which is made up of a series of

rings surrounding a central rod (See Figure 1.1; also MacNab, 2003). At least 20 separate proteins, ranging in copy number from many thousand to a very few, are used to build the flagellar structure itself, and approximately another 30 are used to assist in its construction.

**Figure 1.1. A diagrammatic structure of the *Escherichia coli*/ *Salmonella enterica***

**flagellar paradigm.** The flagellum can be divided into three separate structures, the basal body, hook and filament. The basal body consists of the rod (FlgB, FlgC, FlgF, FlgG and FliE), the rings: MS ring (FliF), P ring (FlgI) and the L ring (FlgH), the stator MotA and MotB), the rotor (FliG), the rotor/ switch apparatus (FliG, FliM and FliN) and the flagellar export apparatus (not shown). The hook consists of the FlgE protein separated by the hook/ filament junction proteins FlgK and FlgL from the filament, which in *E. coli*/ *Salmonella* is a single protein, FliC; the filament is capped by FliD, the capping protein. In *C. jejuni* the filament consists of two proteins, FlaA and FlaB. (Adapted from the KEGG database, Kanehisa *et al.* (2004).



### 1.7.1.1 Basal body

The basal body is situated at the base of the flagellar structure and spans the cytoplasmic membrane, periplasmic space, peptidoglycan layer and outer membrane. It produces torque via the flagellar motor and transmits it to the hook and filament and also anchors the structure into the cell. The basal body consists of the MS-ring situated in the cytoplasmic membrane, a rod that spans the periplasmic space, a periplasmic P-ring and finally an outer membrane L-ring. The flagellar motor has two main components, the stator and the rotor, the stator is made up of multiple copies of the proteins MotA and MotB and is attached non-covalently to the peptidoglycan layer. The rotor, the part which spins, consists of multiple copies of FliG and is non-covalently attached to the MS-ring; it extends into the cytoplasm to form the C-ring. Together these proteins are responsible for generation of the rotary motion of the flagellum by a process which is not entirely understood. In the model proposed by Kojima *et al.* (2001) the MotAB complex harnesses proton movement across the cell membrane and couples this proton flow to the generation of rotational torque in the motor in a process which involves conformational changes induced by the protonation/deprotonation of the Asp32 residue of MotB. The switch, which consists of proteins FliG, FliM and FliN, and allows the motor to switch between clockwise and counter-clockwise rotation in response to environmental signals, is located in the cytoplasm around the C-ring.

### 1.7.1.2 Hook

The hook is situated entirely outside of the cell and connects the basal body to the filament of the flagellum; it is a cylindrical structure composed of multiple copies of a single protein, FlgE. Between the hook and the filament are two short zones of junction proteins,

FlgK and FlgL, which may function as adaptors, allowing the differently structured proteins of the hook and filament to be joined together.

### 1.7.1.3 Flagella filament

The filament is the largest part of a flagellum. It may be up to 20nm in diameter and several microns in length and is composed of tens of thousands of individual flagellin proteins. It is helical in shape and so, when rotated, functions as a propeller-like screw; in the *E. coli*/ *Salmonella* paradigm it is composed of a single type of protein, flagellin, FliC. At the distal end of the filament there is a cap made up of FliD, the filament capping-protein which plays a critical role in the assembly of a growing flagellar filament (Homma *et al.*, 1984). The cap consists of five FliD subunits which form a pentagonal structure with leg-like extended domains which insert individually into cavities at the distal end of the forming flagellum. The space which forms underneath the pentagonal structure allows the flagellin subunits, which have been exported to the distal end of the flagellum, to be added to the structure (Yonekura *et al.*, 2000).

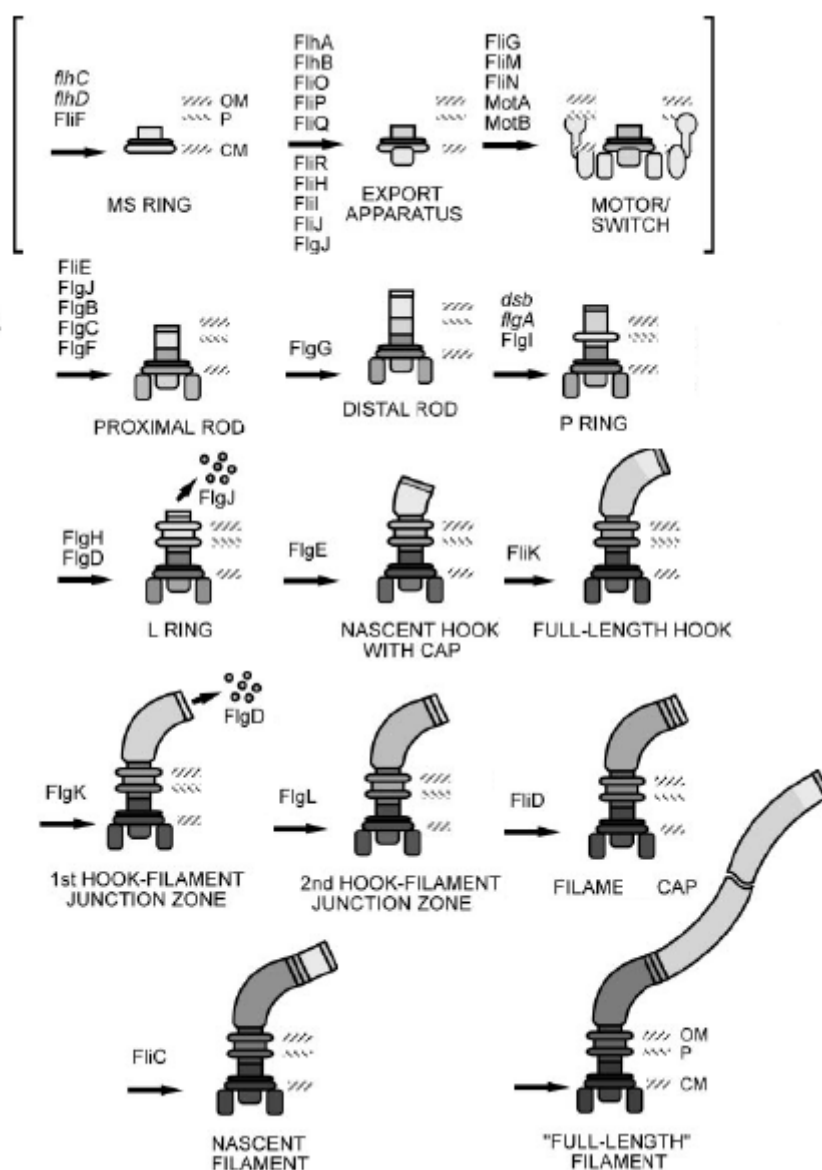
### **1.7.2 Flagellar assembly in *Salmonella***

The assembly of a flagellum is thought to begin with the insertion of the MS-ring into the cytoplasmic membrane via the Sec pathway (see Figure 1.2; Aizawa, 1996). The next event is likely to be the assembly of the C-ring, containing the switch and the Mot proteins, although it has also been hypothesised that the motor and switch proteins are able to insert into the membrane at any time during flagellar formation.

All flagellar proteins assembled after this point use the flagellar type III-like secretion pathway for export, with the exception of the L and P-ring proteins. The remaining proteins of



the basal body are assembled next, beginning with FliE and continuing sequentially, ending with FlgJ, the rod capping protein. The P-ring protein, FlgI, and the L-ring lipo-protein, FlgH, are exported via the Sec pathway. The rod capping protein, FlgJ, is then displaced by FlgD, the hook cap. After this, the addition of hook and filament subunits is only able to take place endogenously, via the type III secretion pathway and the axial channel. The hook is made up of FlgE and its length is controlled by FliK and FlhB, although the mechanism by which this occurs is not entirely understood (MacNab, 2003). Once the hook has reached the correct length, the hook cap is displaced by FlgK, the first hook-filament junction protein; FlgL, the second junction protein is added next. Once FliD, the filament cap is in place, flagellin subunits, FliC, are added underneath it, completing the flagellum.

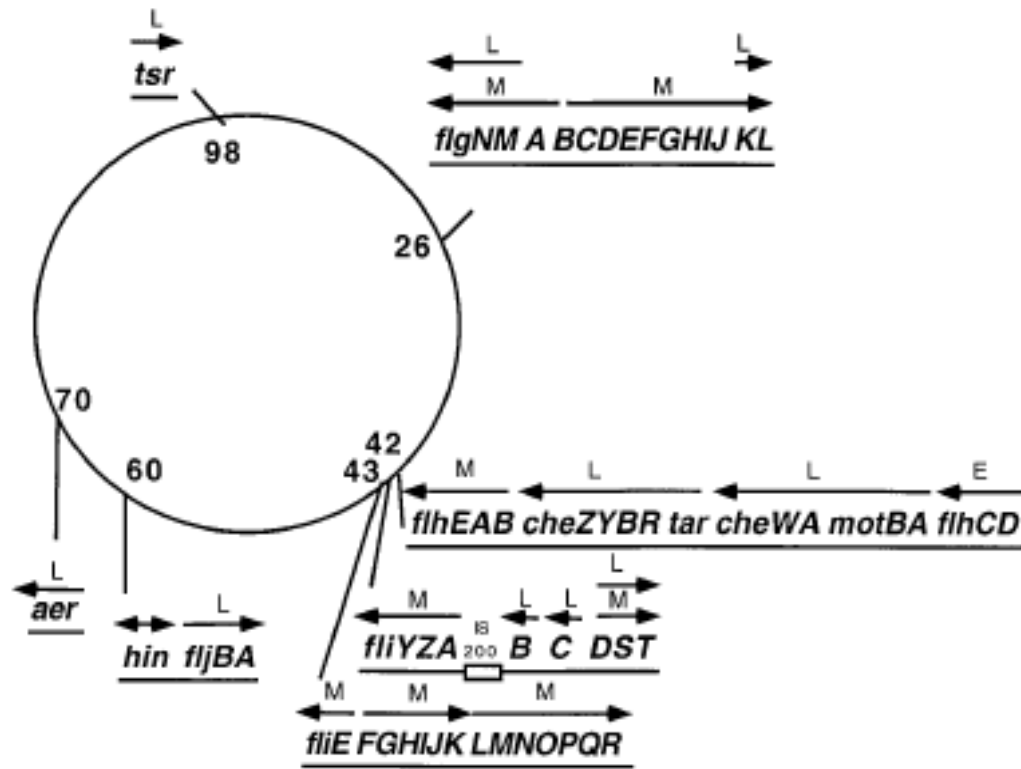


**Figure 1.2. Assembly pathway for the flagellum of *Salmonella*.** Brackets indicate substructures assembled prior to the utilization of the type III export pathway. Genes or proteins necessary at each stage are indicated. CM, cytoplasmic membrane; P, peptidoglycan layer; OM outer membrane. Adapted from MacNab (2003)

### 1.7.3 Flagellar gene organisation in *E. coli*/*Salmonella*

More than 50 genes are required for the assembly and function of the flagellum in the *E. coli*/*Salmonella* model; these include structural subunits, regulatory proteins, motor proteins and chemosensory mechanisms (Chilcott and Hughes, 2000). The nomenclature of the majority of these genes begins with *fl* and is followed by either *g*, *h* or *i* depending upon the location of the gene on the *E. coli*/*Salmonella* chromosome, a final letter, beginning with *A*, is added depending upon the flagellar gene organisation in that region of the chromosome (Iino *et al.*, 1988). Whilst this is true for the majority of genes in the *E. coli*/*Salmonella* model, some genes were identified after this system had been established and others are named for their null phenotype, e.g. *motA* and *motB* for motility negative.

In the *E. coli*/*Salmonella* model, motility and chemotaxis genes are mainly arranged in clusters in three chromosomal regions (See Figure 1.3; also Macnab, 1996; Soutourina and Bertin, 2003). Cluster I consists of the *flg* structural genes, cluster II the *flh* regulator, motor and chemotaxis genes and cluster III, the *fli* structural and export apparatus genes; this region consists of two parts, separated by a smaller region of DNA unrelated to the flagellar genes. Flagellar gene organisation differs considerably across the various genera that possess flagella, with some organisms having quite coherently arranged operons, such as *Pseudomonas aeruginosa* (Dasgupta *et al.*, 2003); others have apparently little organisation, such as *C. jejuni* (Parkhill *et al.*, 2000).



**Figure. 1.3.** Chromosomal locations of the operons that make up the flagellar regulon of *Salmonella enterica* serovar Typhimurium. The operons are labelled E, M, or L for early, middle or late expression respectively. Arrows indicate co-expression of a group of genes to form an operon. Adapted from Chilcott and Hughes (2000).

#### 1.7.4 Flagellar gene expression in *E. coli*/ *Salmonella*

In Gram-negative bacteria flagellar gene expression is hierarchical, with genes expressed in the order in which they are required for flagellar assembly and controlled by transcriptional regulators, including alternative sigma factors and anti-sigma factors (Macnab, 2003; Soutourina and Bertin, 2003). Genes are divided into three hierarchical transcription classes, early, middle and late (see Figure 1.3). The early genes are regulatory proteins that may control the expression of the entire regulon; middle genes generally produce the structural components of the basal body, hook and associated type III secretion system. Products of late genes include the filament, some components of the motor system and chemosensory proteins (Aldridge and Hughes, 2002). As some of the genes or clusters in each group are expressed from different promoters, the promoters themselves are organised into three classes (Chilcott and Hughes, 2000). Early genes typically have class I promoters, middle genes class II and late genes class III.

The *flhDC* master switch operon is at the top of the hierarchy, as FlhD and FlhC are required for the expression of all other regulatory genes responsible for flagellar biosynthesis. The cyclic AMP catabolite activator protein (cAMP-CAP) is an activator of the *flhDC* master operon and hence the operon is sensitive to environmental and cell state signals. FlhD and FlhC form a heterotetrameric complex, which is responsible for the transcription of certain middle and late genes from  $\sigma^{70}$  dependant promoters (Chilcott and Hughes, 2000). Proteins in the middle class of expression are mainly structural proteins and those involved in assembly of the hook-basal body of the flagellum. Also transcribed are transcriptional regulators FliA, an alternative sigma factor,  $\sigma^{28}$ , required for transcription of late flagellar genes and FlgM, an anti-sigma factor which negatively regulates late genes (Ohnishi *et al.*, 1992). Before formation of the flagellar secretory apparatus FlgM is present in the cytoplasm and is

complexed with  $\sigma^{28}$ , inhibiting the association of  $\sigma^{28}$  with RNA polymerase and hence transcription. After hook-basal body synthesis, which includes synthesis of the type III-like secretion system, FlgM is secreted, allowing  $\sigma^{28}$  to bind RNA polymerase and for transcription of the late genes to occur (Hughes *et al.*, 1993). Certain genes are transcribed from both FlhDC class II and  $\sigma^{28}$  dependent class III promoters, such as *fliD*, the filament cap gene, and *fliK*, the hook length control protein; as such they are expressed at both the middle and late stages of flagellar biosynthesis (Kutsukake 1994).

#### **1.7.5 Differences between flagella in *C. jejuni* and the *E. coli*/ *Salmonella* paradigm.**

*C. jejuni* differs from both *E. coli* and *Salmonella* in that it has only a single flagellum at one or both ends of the cell. More bacteria are thought to possess these polar flagella than the peritrichous flagella of the *E. coli*/ *Salmonella* model, although this latter system has been studied in more detail. There are several major differences between the two systems; one of the most notable is the absence of the *flhDC* master regulator system in most non-enterobacteriaceae. There are also observable differences in the quaternary structure of *C. jejuni* and *Salmonella* flagella; *C. jejuni* flagella are heavily glycosylated whilst *E. coli* and *Salmonella* flagella are unglycosylated. Hence it is clear that the *E. coli*/ *Salmonella* paradigm is not sufficient for the understanding of all flagellar systems and that other models of bacterial flagellar systems are required.

##### 1.7.5.1 Flagellar genes in *C. jejuni*.

Approximately 42 genes (Parkhill *et al.*, 2000), are thought to be involved in flagellar biosynthesis in *C. jejuni*, the majority of which are conserved with those of the *E. coli*/ *Salmonella* paradigm (Jagannathan and Penn, 2005); *C. jejuni* flagellar genes have been

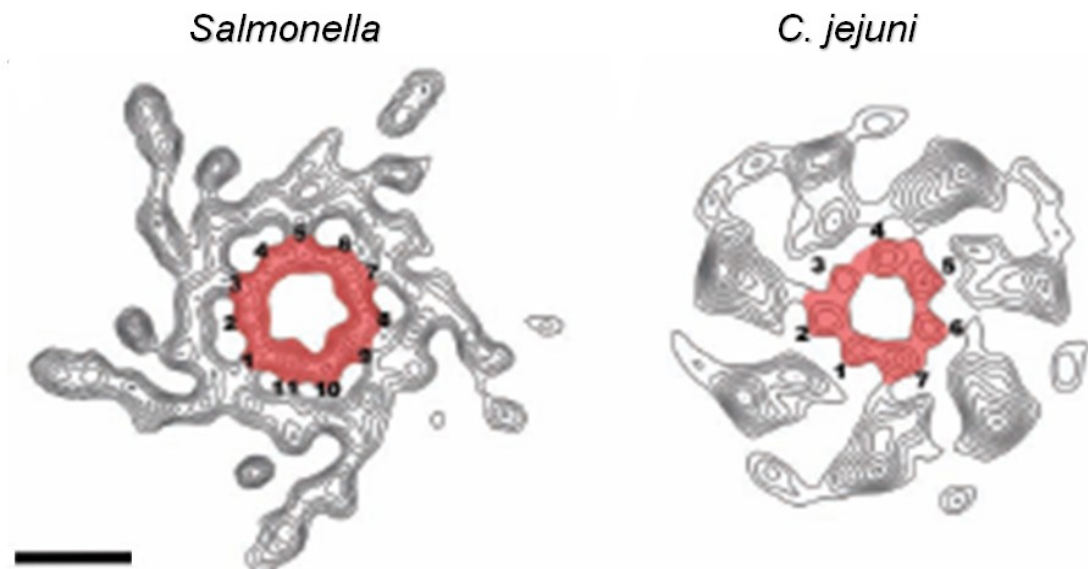
named based on their similarities to *E. coli*/*Salmonella* genes. Whilst in many bacteria flagella genes are clustered in defined regions, those of *C. jejuni* are distributed throughout the genome at 32 different loci (Guerry *et al.*, 2000; Parkhill *et al.*, 2000). If early *C. jejuni* flagellar genes were regulated and expressed together with genes of other functions, or were expressed constitutively, the absence of a master regulator in this species could, at least in part, be explained. Genes found in the *E. coli*/*Salmonella* model and missing in *C. jejuni* include the *flhDC* master regulators, anti- $\sigma^{28}$  factor *flgM*, and several chaperones and rod-hook associated proteins (Parkhill *et al.*, 2000). Genes encoding additional proteins are also present; *rpoN* encodes  $\sigma^{54}$ , a principle element in flagellar gene expression control. Also an NtrC homologue, FlgR, has been shown to act as a transcriptional activator of  $\sigma^{54}$  dependent genes (Jagannathan *et al.*, 2001). Other genes present include a two-component regulator system comprising FlgR-FlgS (Hendrixson and DiRita, 2003) and a duplication of *flgE*, the hook structural protein, amongst others (Jagannathan and Penn, 2005). In *C. jejuni* there are also approximately 50 genes involved in flagellar glycosylation which are entirely absent from *E. coli* and *Salmonella*. The exact function of flagellar glycosylation is unknown but it is apparent that it has some role in flagellar biogenesis and function and also probably is involved in either bacteriophage or host immune system evasion.

#### 1.7.5.2 Flagellar structure in *C. jejuni*

The structural parameters of *Salmonella* flagella have been closely measured; filaments are usually 15 $\mu$ m long, 220Å wide, are formed of 11 protofilaments and comprise of approximately 30,000 FliC subunits (Macnab, 1996). Protofilaments are strands formed of stacked flagellin subunits and 11 of these stacks, arrayed helically, are needed in *Salmonella* to form the tubular flagellar filament. Protofilaments can exist in two slightly different

conformations, left and right-handed which alter the repeat distance and packing of the protofilaments altering the superstructure of the flagellum (O'Brien and Bennett, 1972). Different arrangements of protofilaments can produce two straight and 10 types of supercoiled flagellar filament (Calladine, 1975). The helical structure of a flagellum is necessary to allow the flagellum to generate thrust and propel the cell when rotated; an entirely straight flagellum is unable to do so. Given the sequence similarity between the flagellar filament genes of *C. jejuni* (FlaA and FlaB) and *Salmonella* (FliC) it has been generally assumed that *C. jejuni* and other bacterial flagella are likely to be composed of 11 protofilaments. However, Galkin *et al.* (2008) using electron microscopy identified that flagella from *C. jejuni* were in fact narrower than those from *Salmonella*, approximately 180Å rather than 220Å. Given that *C. jejuni* flagellins have a greater molecular weight (59.5kDa alone or approximately 65kDa including glycosylations) than *Salmonella* FliC (52.2kDa), this result was surprising. Further work by this group demonstrated that in fact *Campylobacter* flagella have only seven protofilaments, which are more loosely packed resulting in a lumen of a comparable size, Figure 1.4.

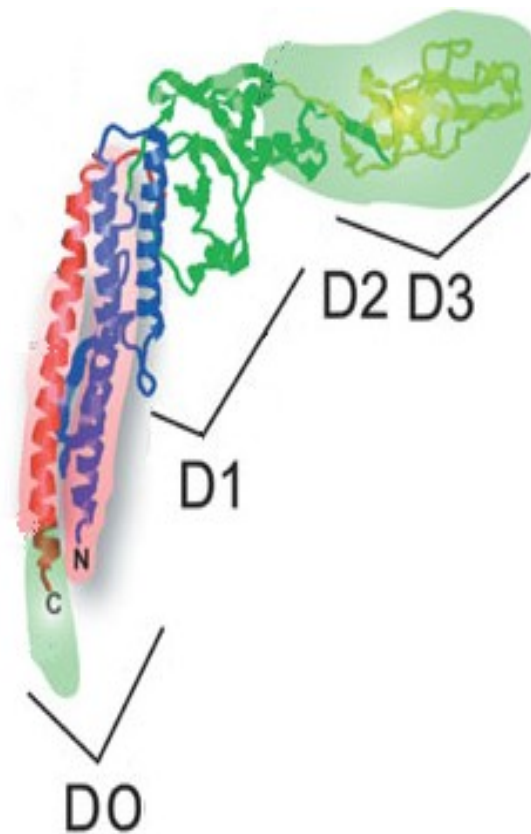




**Figure 1.4.** Three dimensional reconstructions of flagellar filaments of *Salmonella* and *C. jejuni*. Showing the central lumen (coloured red) surrounded by 11 protofilaments in *Salmonella* and seven in *C. jejuni*. The Scale bar indicates 50 Å. Adapted from Galkin *et al.* (2008).

Another major difference between *Salmonella* and *C. jejuni* flagella is that *C. jejuni* flagellin, like flagellin from the other epsilon-Proteobacteria, does not activate vertebrate Toll-like receptor 5 (TLR5; Andersen-Nissen *et al.*, 2005). Toll-like receptors are an important family of innate immune receptors that recognise pathogen associated molecular patterns. These are usually evolutionarily conserved structures, vital for microbial fitness, that are not present in the host (Andersen-Nissen *et al.*, 2007). TLR5 is known to recognise bacterial flagellin (Hayashi *et al.*, 2001) and the recognition site is located in the highly conserved D1 domain of the flagellin protein (Smith *et al.*, 2003), although the D2 and D3 domains also appear to be required. No post-translational modifications are thought to be involved. This domain, at least in *Salmonella*, interacts with adjacent flagellin monomers via polar-polar and charged-polar interactions to form the flagellar filament (Yonekura *et al.*, 2003); the structure of the *Salmonella* flagellin protein, FliC, is shown in Figure 1.5. In *Salmonella*, mutants in individual residues in the TLR5 recognition site had either severely reduced motility or were entirely non-motile (Smith *et al.*, 2003), indicating that the residues recognised by TLR5 are also likely to be required for correct flagellin subunit-subunit interactions

Work by Andersen-Nissen *et al.* (2005) showed that *C. jejuni* flagellin was not recognised by TLR5 and identified several specific changes in the TLR5 recognition site residues that are responsible for TLR5 evasion. Whilst these changes in structure, if present in *Salmonella* flagellin, would cause a decrease in motility, *C. jejuni* flagellin possesses at least some compensatory mutations that allow for the flagellin subunit interactions necessary for motility, but which are not involved in TLR5 recognition. Galkin *et al.* (2008) suggest that the altered protofilament arrangement, and consequent alteration in the packing of D1 domains, which they discovered in *C. jejuni* may be a product of mutations which led to the ability of *C. jejuni* to evade TLR5 recognition.



**Figure 1.5. Structure of the *Salmonella* flagellin FliC, showing the four domains.**

Domains D0 and D1 are well conserved between different bacterial species and in the flagellum are helically stacked to form the filament core. Domains D2 and D3 are highly variable in both sequence and length between different bacterial species and are surface exposed, protruding from the side of the flagellar filament. Adapted from Samatey *et al.* (2001).

## 1.8 Protein glycosylation in *C. jejuni*

Once believed to occur only in eukaryotes, it is now evident that glycoproteins are a common feature in all domains of life. Information about the structures of linked glycans and the process of glycosylation is limited, although it appears that bacterial glycans are more complex than those found in eukaryotes. One of the best studied examples of bacterial protein glycosylation is that of *C. jejuni* where two glycosylation loci have been identified which encode both *O*-linked and *N*-linked protein glycosylation pathways.

### 1.8.1 *N*-linked protein glycosylation

The *N*-linked general protein glycosylation system was thought, when the gene cluster was first discovered, to be involved in the biosynthesis of lipopolysaccharide (Korolik *et al.*, 1997). Subsequently it was shown, by Szymanski *et al.* (1999), to have a role in the glycosylation of a multitude of *C. jejuni* proteins. The *N*-linked glycosylation locus is 16kb in size and genes from this locus are named *pgl* for protein glycosylation. The structure of the glycans, which are heavily modified bacillosamine residues, has been deduced, the glycans are linked to proteins through asparagine residues in the motif Asn-Xaa-Ser/Thr, which is the same motif used in eukaryotes (Young *et al.*, 2002; Wacker *et al.*, 2002). Glycosylation of proteins occurs in the periplasm and more than 30 proteins are believed to be glycosylated by this locus (Young *et al.*, 2002), the majority of which appear to be either surface exposed or secreted. Unlike the genes involved in *C. jejuni* flagellin glycosylation, the *pgl* locus is highly conserved between strains of *C. jejuni* and *C. coli* and lacks homopolymeric sequences (Szymanski *et al.*, 2003). This suggests that the generation of structural and antigenic diversity is not the primary role of this system, which remains unclear, although as the

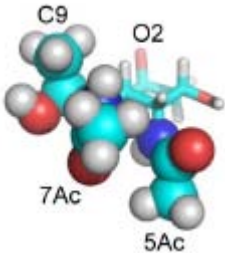
majority of proteins modified by this system are surface-exposed or excreted, it is possible the protection against extracellular proteases could be an important role for this system.

### 1.8.2 O-linked flagellin glycosylation

Initial evidence for the post-translational modification of *Campylobacter* flagellin came from the analysis of *C. coli* VC167 flagellin by Logan *et al.* (1989) who identified a number of serine residues in the central region of the amino acid sequence that were modified. That glycosylation was the post-translational modification involved was originally suggested by Doig *et al.* (1996) who demonstrated that *C. jejuni* flagellins are sensitive to periodate oxidation and could bind sialic-acid-specific lectins. The subsequent sequencing of the *C. jejuni* NCTC 11168 genome by Parkhill *et al.* (2000) revealed the presence of a flagellar glycosylation gene cluster containing almost 50 genes, the majority of which are not shared with *H. pylori* (Jagannathan and Penn, 2005). The genes encoding the flagellin proteins FlaA and FlaB are the only flagellar structural genes included in this cluster, which has been designated the flagellin glycosylation locus, see Figure 1.8 and Table 1.2. Annotation of the glycosylation locus predicted that approximately half of the genes in this region would have a role in glycan biosynthesis (Parkhill *et al.*, 2000). The size of the locus varies considerably between strains, from the 20kb locus of *C. jejuni* 81-176 to the 50kb locus of *C. jejuni* NCTC 11168, which is the largest known. It was initially supposed that the sugar biosynthesis genes in the flagellin glycosylation locus were involved in sialic acid biosynthesis (Doig *et al.*, 1996). It was later realised, through the analysis of flagellins in *C. jejuni* by Thibault *et al.* (2001) and in *C. coli* by Logan *et al.* (2002), that the modification on the flagellins was in fact pseudaminic acid (Pse), a structural relative of sialic acid. Pse is a nine-carbon sugar member of the 5, 7-diamino-3, 5, 7, 9-tetradexynon-2-ulosonic acid family; details of all of the

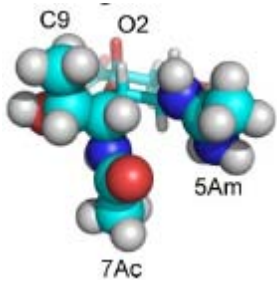
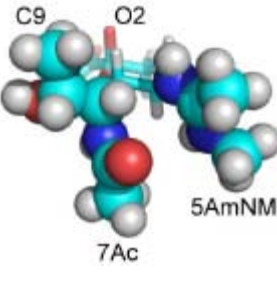
flagellar glycans thus far identified in *Campylobacter* are given in Table 1.1. So far investigations have focused on *C. coli* VC167 and *C. jejuni* 81-176 (examples include: Thibault *et al.*, 2001 and McNally *et al.*, 2007) as these strains have smaller flagellin glycosylation loci and it was hypothesised that their post-translational glycosylations would be correspondingly simpler.

**Table 1.1 Post-translational modifications of *Campylobacter* flagellins.** Structures adapted from McNally *et al.* (2007). The major modifications present in the various strains are indicated by \*.

Modification	Structure	Neutral Glycan mass (Da)	Glycan residue mass (Da) <sup>d</sup>	Present on flagellins of:		
				<i>C. jejuni</i> 81-176	<i>C. coli</i> VC167	<i>C. jejuni</i> NCTC 11168
Pseudaminic acid (Pse5Ac7Ac or PseAc)		334	316	✓ <sub>b*</sub>	✓ <sub>a</sub>	✓ <sub>c</sub>
Acetamidino pseudaminic acid (Pse5Ac7Am or PseAm)	-	333	315	✓ <sub>b*</sub>	-	✓ <sub>c</sub>
<i>O</i> -acetyl pseudaminic acid (Pse5Ac7Ac8OAc or PseOAc)	-	376	358	✓ <sub>b</sub>	-	-

N-acetyl glutamine pseudaminic acid derivative (Pse5Ac7Ac8-GlnAc or PseGlnAc)	-	504	486	✓ <sub>b</sub>	-	-
Dihydroxypropionyl pseudaminic acid derivative (PsePr)	-	427	409	✓ <sub>b</sub>	-	-
Dimethylglyceric derivative of acetamidino pseudaminic acid	-	405	389	-	-	✓ <sub>c*</sub>
Dimethylglyceric derivative of pseudaminic acid	-	406	390	-	-	✓ <sub>c*</sub>
Acetamidino pseudaminic acid-deoxypentose	-	449	431	-	✓ <sub>c</sub>	-
O-acetyl pseudaminic acid-deoxypentose	-	450	432	-	✓ <sub>c</sub>	-



Legionaminic acid derivative (Leg5Am7Ac or LegAm)		333	315	-	✓ <sub>a*</sub>	✓ <sub>c</sub>
Legionaminic acid derivative (Leg5AmNMe7Ac or MeLegAM).		347	329	-	✓ <sub>a*</sub>	✓ <sub>c</sub>

Identified by:

<sup>a</sup> McNally *et al.* (2006a);

<sup>b</sup> Thibault *et al.* (2001);

<sup>c</sup> Logan *et al.* (2009).

<sup>d</sup> The glycan residue mass gives the mass of the glycan minus the mass of one H<sub>2</sub>O molecule, which is as it would appear if identified from flagellar glycopeptides analysed by mass spectrometry.

### 1.8.2.1. Flagellin glycosylation in *C. jejuni* 81-176.

Flagellins from *C. jejuni* 81-176 were examined by Thibault *et al.* (2001) and found to be modified at 19 serine and threonine residues, see Figure 1.6; the modifications together contribute approximately 10% (6000Da) to the molecular mass of the flagellin. Pseudaminic acid (PseAc) was identified as the major glycosyl component, while other modifications structurally related to PseAc are present and include acetamidino pseudaminic acid (PseAm), an *O*-acetyl derivative of PseAc (PseOAc) and a dihydroxypropionyl derivative, (PsePr), see Table 1.1. It was shown that only selected serine and threonine residues were glycosylated and that the modifications themselves appeared to be restricted to particular residues, suggesting that the glycosylation process is specific. However, it was also noted that in a *pseA* mutant that was unable to produce PseAm, the residues which would have been modified with PseAm were instead modified with PseAc. Most of the modified residues are located in a narrow region of the central flagellin domain, corresponding to domains D2 and D3 in *Salmonella* flagellin, which are believed to be surface exposed (Power *et al.*, 1994; Logan *et al.*, 2008); no evidence of the presence of unglycosylated flagellin was found.

There are 23 flagellin glycosylation associated genes in *C. jejuni* 81-176, the best characterised of which are those of the *pse* family, which form the pathway by which *C. jejuni* 81-176 synthesises and attaches PseAc and PseAm to flagellin (Guerry *et al.*, 2006); the means by which PseOAc and PsePr are synthesised are unclear. The full biosynthetic pathway for the conversion of UDP-N-acetylglucosamine to pseudaminic acid involves PseB/Cj1293, PseC/Cj1294, PseH/Cj1313, PseG/Cj1312, PseI/Cj1317 and PseF/Cj1311. All *pse* genes thus far identified have homologues in *C. jejuni* NCTC 11168, see Table 1.2.

81176 MGFRINTNVAALNAKANSDLNAKSLDASLSRLSSGLRINSAADDASGMAIADSLRSQANT 60

81176 LGQAISSNGNDALGILQTADKAMDEQLKILDTIKTKATQAAQDGQSLKTRTMLQADINKLM 120

81176 EELDNIANTTTSFNGKQLLSGNFTNQEFQIGASSNQTVKATIGATQSSKIGVTRFETGAQS 180

81176 FTSQGVVGLTIKNYNGIEDFKFDNVVISSTSVGTGLGALAEINKSADKTGVRATYDVKTG 240

81176 VYAIKEGTTTSQEFANGVTIGKIEYKDGNGSLISAINAVKDTTGVQASKDENGKLVLT 300

81176 SADGRGIKITGDIGVSGILANQKENYGRSLVKNDGRDINISGTNLSAIGMGTTDMISQ 360

81176 SSVSLRESKGQISATNADAMGFNSYKGGGKFVFTQNVSSISAFMSAQSSGFSRSGFSVG 420

81176 SGKNLSVGLSQGIQIISAAASMSNTYVVSAGSGFSSGSGNSQFAALKTTAANTTDETAGV 480

81176 TILKGAMAVMDIAETAITNLDQIRADIGSIQNQVTSTINNITVTQVNVKAAESQIRDVDF 540

81176 ASESANYSKANILAQSSGYAMAQANSSQQNVLRLLQ 576

**Figure 1.6. Locations of glycosyl modifications on *C. jejuni* 81-176 flagellin (Thibault *et al.*, 2001).** Residues shown to be modified with pseudaminic acid derived glycosyl groups are shown in red and other serine and threonine residues are shown in yellow.

#### 1.8.2.2. Flagellin glycosylation in *C. coli* VC167.

Flagellin modification in *C. coli* VC 167 was first examined by Logan *et al.* (2002), who showed that flagellins from this strain were post-translationally modified at more than 16 amino acid residues. The locations of the modifications are shown in Figure 1.7 and the details of the sugars in Table 1.1. Both PseAc and what was initially thought to be a form of PseAm slightly different in structure to that found on *C. jejuni* 81-176 flagellin were identified; PseOAc and PsePr were not found. McNally *et al.* (2007) discovered that the form of PseAm was in fact likely to be a legionaminic acid derivative with the same mass. Legionaminic acid is a 9-carbon sugar, previously identified as part of the lipopolysaccharide of *Legionella pneumophila* and *Pseudomonas aeruginosa* (Luneberg *et al.*, 2000; Knirel, 2003); two legionaminic acid derivatives actually present on *C. coli* VC167 flagellins are, the acetamidino form, LegAm, and an *N*-methyleacetimidoyl form, MeLegAm (McNally *et al.*, 2007).

The flagellar glycosylation region of *C. coli* VC167 contains 14 genes including genes homologous to those of the *pse* family of *C. jejuni* 81-176, although in this strain *pseA*, which synthesises PseAm directly from PseAc in *C. jejuni* 81-176, is a pseudogene. *C. coli* VC167 also has a separate group of genes, the *ptm* family (post-translational modification), which are involved in the synthesis of the legionaminic acid derivatives.

0 GFRINTNVAA LNAKANSDLN SRALDQSLSR LSSGLRINSA ADDASGMAIA  
 51 DSLRSQANTL GQAI SNGNDA LGILQTADKA MDEQLKILD T IKTKATQAAQ  
 101 DGQSLKTRTM LQADINRLME ELDNIANTTS FNGKQLLSGG FTNQEFQIGS  
 151 SSNQTIKASI GATQSSKIGV TRFETGSQSF SSGTVGLTIK NYNGIEDFKE  
 201 QNVVI STSVG TGLGALAEI NRNADKTGIR ATFDVKSVGA YAIKAGNTSQ  
 251 DFAINGVVIG KVDY SDGDEN GSLISAINAV KD TTGVQASK DENGKLVLT  
 301 ADGRGIKITG SIGVGAGILH TENYGRLSLV KNDGRDINIS GTGLSAIGMG  
 351 ATDMISQSSV SLRESKGQIS AANADAMGFN AYNGGGAKQI IFASSIAGFM  
 401 SQAGSGFSAG SGFSVSGKN YSAIL SASIQ IVSSAASS TYVVSTGSGF  
 451 SAGSGNSQFA ALRISTVSAH DETAGVTTLK GAMAVMDIAE TAITNLDQIR  
 501 ADIGSVQNQI TSTINNITVT QVNVKSAESQ IRDVDFASES ANYSKANILA  
 551 QSGSYAMAQA NSSQQNVRLRL LQ

**Figure 1.7. Locations of glycosyl modifications on *C. coli* VC167 flagellin (Logan *et al.*, 2002).** Peptides examined for modified residues are underlined and residues known to be definitely modified are shown red, while other serine and threonine residues are shown in yellow

### 1.8.2.3. Flagellin glycosylation in *C. jejuni* NCTC 11168.

Whilst homologues of genes from both the *pse* and *ptm* pathways are present in the flagellin glycosylation region of *C. jejuni* NCTC 11168, many other genes in this region remain hypothetical; the genes of this region are detailed in Table 1.2. The types of modifications present on *C. jejuni* 11168 flagellin have been examined by Logan *et al.* (2009), but none have been located to specific residues. As the genes of the flagellin glycosylation region of *C. jejuni* 11168 have homologues in the glycosylation regions of *C. jejuni* 81-176 and *C. coli* VC167 it is unsurprising that *C. jejuni* 11168 flagellin should be glycosylated with some of the carbohydrates found on flagellins from these strains including PseAc, PseAm and legionaminic acid derivatives (Logan *et al.*, 2009). But the increased complexity of the glycosylation locus in this strain also suggests that a wider variety of modifications, resulting from several different pathways could also be present. As such, Logan *et al.* (2009) also identified di-O-methylglyceric derivatives of both PseAc and PseAm which had not been identified previously in *Campylobacter* and were the most abundant modifications present.

Whole genome comparisons have identified the flagellar locus as one of seven hypervariable plasticity regions in the *Campylobacter* genome (Pearson *et al.*, 2003). The genes of the *C. jejuni* NCTC 11168 flagellar glycosylation region are detailed in Table 1.2, together with their proposed functions and details of homologous genes in *C. jejuni* 81-176 and *C. coli* VC167. Some of these genes are members of the 1318 motility accessory factor (1318/*maf*) family of flagellin-associated proteins. The *maf* family has seven members, Cj1318, *pseD* (Cj1333), Cj1334, Cj1335/6, *pseE* (Cj1337), Cj1340 and Cj1341, several of which are involved in motility variation by slipped-strand mispairing of homopolymeric tracts (Karlyshev *et al.*, 2002). *maf2* (Cj1333) has recently been re-annotated as *pseD* and has been shown to have some involvement in the attachment of

PseAm to flagellin in *C. jejuni* 81-176 (Guerry *et al.*, 2006). *maf5* (Cj1337) has likewise been re-annotated as *pseE* (McNally *et al.*, 2006a); the functions of the other members of the *maf* family are unknown, although as both *pseD* and *pseE* have been implicated in the attachment or transfer of sugar groups to flagellin it is possible that all genes in this family have roles in the later stages of flagellin glycosylation if all are not involved in attachment. The proteomic map produced by Parrish *et al.* (2007) indicated that PseE interacts directly with FlaA and FlaB, further supporting its assignment as a gene involved in sugar transfer or attachment.

A comparative phylogenomic study by Champion *et al.* (2005) identified a cluster of genes (Cj1321 to Cj1326) as being characteristic of a livestock clade. Recently Howard *et al.* (2009) confirmed that the genes of this locus, especially Cj1324, have a significant part in promoting the ability of *C. jejuni* to colonise chickens. Mutants in Cj1324 (*ptmG*), which were motile and retained full-length flagella, were also less hydrophobic and less able to autoagglutinate and form biofilms than the wild-type. Analysis of Cj1324 mutant flagellin also showed that certain legionaminic acid derivatives were absent from flagellins, subsequently, these particular glycans were shown to be prevalent in *Campylobacter* strains isolated from chickens, providing strong evidence for the involvement of flagellin glycosylation in colonisation and explaining why certain *Campylobacter* isolated are more likely to colonise chickens than others (Howard *et al.*, 2009).

*maf1* and *maf4* are direct repeats, each containing a homopolymeric sequence; another homopolymeric tract is located in *maf7* (Cj1342c). Altogether the flagellin glycosylation locus includes 10 homopolymeric sequences, nine of which are located within the coding region of a gene (Turner and Penn, 2005). In *Campylobacter* these tracts have been seen to vary in length at a frequency higher than that found in other

organisms; some regions of the *C. jejuni* genome show two or three sequence variants present simultaneously in similar amounts, which is not often seen in other organisms (Parkhill *et al.*, 2000).

#### 1.8.2.4 Flagellin glycosylation in *Helicobacter pylori*

*Helicobacter pylori*, a mucosal pathogen and closely related species to *Campylobacter* also produces flagellins that are glycosylated with PseAc. In this bacterium it appears that both the FlaA and FlaB proteins are glycosylated, with seven and ten residues respectively (Schirm *et al.*, 2003); in *Campylobacter* it has never been possible to determine whether both flagellins are glycosylated due to their high sequence similarity. Only the single glycan, PseAc, has been observed decorating *H. pylori* flagellin, this is reflective of the limited number of flagellin glycosylation related genes present in *H. pylori* in comparison to *Campylobacter* (Josenhans *et al.*, 2002; Parkhill *et al.*, 2000). Glycosylation appears, as in *Campylobacter*, to be necessary for flagellar assembly in *Helicobacter* (Schirm *et al.*, 2003). The presence of a sheath covering the flagellar filament may reduce the need for a great diversity in the types of flagellar glycans on *H. pylori* flagellin, particularly if the glycans are involved in host immune system or bacteriophage evasion.



**Table 1.2. The flagellin glycosylation locus of *C. jejuni* NCTC 11168.** Genes shown in bold are part of the flagellar modification region (FMR). c indicates that the coding sequence of the gene is located on the complementary strand.

Gene	Homolog in		Motility of mutant in <i>C. jejuni</i> 81-176	<i>maf</i> family genes	Homopolymeric tract <sup>c</sup>	Function
	<i>C. jejuni</i> 81-176	<i>C. coli</i> VC167				
<i>pseB</i> (Cj1293)	✓	-	Mot-	-	-	The first stage in PseAc synthesis, the mutant accumulates unglycosylated flagellin monomers intracellularly <sup>1</sup>
<i>pseC</i> (Cj1294)	✓	-	Mot-	-	-	PseAc biosynthesis <sup>2</sup>
Cj1295	✓	-	Mot+	-	G(9)	Unknown
Cj1296	-	-	-	-	G(9)	Unknown
Cj1297	-	-	-	-		Unknown
Cj1298	✓	-	Mot+	-	-	Unknown
Cj1299	-	-	Mot+	-	-	Unknown
Cj1300	✓ -on c strand	-	Mot+	-	-	Unknown
Cj1301	-	-	-	-	-	Unknown
Cj1302	✓	-	Mot+	-	-	Unknown
<i>fabH2</i> (Cj1303)	✓	-	Mot+	-	-	Putative acyl-carrier-protein synthase
<i>accP</i> (Cj1304)	✓	-	Mot+	-	-	Putative acyl-carrier-protein
Cj1305c	✓	-	Mot+	-	C(9)	Unknown
Cj1306c	-	-	-	-	C(9)	Unknown
Cj1307	-	-	-	-	-	Unknown

Cj1308	-	-	-	-	-	Unknown
Cj1309c	-	-	-	-	-	Unknown
Cj1310c	-	-	-	-	C(9)	Unknown
<i>pseF</i> (Cj1311)	✓	-	Mot-	-	-	PseAc biosynthesis <sup>2</sup>
<i>pseG</i> (Cj1312)	✓	-	Mot-	-	-	PseAc biosynthesis <sup>3</sup>
<i>pseH</i> (Cj1313)	✓	-	Mot-	-	-	PseAc biosynthesis <sup>3</sup>
<i>HisH</i> (Cj1314c)	✓	✓	Mot+	-	-	Histidine biosynthesis <sup>2</sup>
<i>HisF</i> (Cj1315c)	✓	✓	Mot+	-	-	Histidine biosynthesis <sup>2</sup>
<i>pseA</i> (Cj1316c)	✓	✓ -pseudogene <sup>b</sup>	Mot+	-	-	Synthesis of PseAm directly from PseAc, a mutant in <i>C. jejuni</i> 81-176 was motile but had all PseAm modifications replaced with PseAc <sup>4</sup> . Is a pseudogene in <i>C. coli</i> VC167
<i>pseI</i> (Cj1317)	✓	✓	Mot-	-	-	Final stage of PseAc synthesis in <i>C. jejuni</i> 81-178 <sup>5</sup>
<b>Cj1318</b>	-	-	-	<i>mafI</i>	<b>G(11)</b>	<b>Unknown</b>
<b>Cj1319</b>	-	✓	-	-	-	<b>Unknown</b>
<b>Cj1320</b>	-	✓	-	-	<b>Integenic G(10-11)</b>	<b>Unknown</b>
<b>Cj1321</b>	-	-	-	-		<b>Unknown</b>
<b>Cj1322</b>	-	-	-	-	-	<b>Unknown</b>
<b>Cj1323</b>	-	-	-	-	-	<b>Unknown</b>
<i>ptmG</i> (Cj1324)	-	✓	-	-	-	<b>Legionaminic acid biosynthesis<sup>6</sup></b>
<i>ptmH</i> (Cj1325/6) <sup>d</sup>	-	✓	-	-	<b>G(10)</b>	<b>Legionaminic acid biosynthesis<sup>6</sup></b>
<i>ptmC</i> (Cj1327)	-	✓	-	-	-	<b>Legionaminic acid biosynthesis<sup>7</sup></b>
<i>ptmD</i> (Cj1328)	-	✓	-	-	-	<b>Legionaminic acid biosynthesis<sup>6</sup></b>
<i>ptmE</i> (Cj1329)	-	✓	-	-	-	<b>Legionaminic acid biosynthesis<sup>6</sup></b>

<i>ptmF</i> (Cj1330)	-	✓	-	-	-	Legionaminic acid biosynthesis <sup>6</sup>
<i>ptmB</i> (Cj1331)	-	✓	-	-	-	Legionaminic acid biosynthesis <sup>7</sup>
<i>ptmA</i> (Cj1332)	-	✓	-	-	-	Legionaminic acid biosynthesis <sup>6</sup>
<i>pseD</i> (Cj1333)	✓	-	Mot+	<i>maf2</i>	-	Involved in attachment of PseAm to flagellin <sup>2,3</sup>
Cj1334	✓	-	Mot+	<i>maf3</i>	-	Unknown
Cj1335/6 <sup>d</sup>	-	-	-	<i>maf4</i>	G(9)	Unknown; in <i>C. jejuni</i> 108 a functional <i>maf4</i> gene caused novel flagellin glycoforms and increased autoagglutination <sup>8</sup>
<i>pseE</i> (Cj1337)	✓	-	Mot-	<i>maf5</i>	-	Involved in assembly and transfer of glycosyl groups to flagellin <sup>3,9</sup>
<i>flaB</i> (Cj1338c)	✓	✓	-	-	-	Minor flagellin protein
<i>flaA</i> (Cj1339c)	✓	✓	-	-	-	Major flagellin protein
Cj1340c	-	-	-	-	-	Unknown
Cj1341c	✓	-	Mot+	<i>maf6</i>	-	Unknown
Cj1342c	✓	-	Mot+	<i>maf7</i>	C(9)	Unknown

<sup>a</sup> - In *C. jejuni* 81-176 the Cj1300 coding sequence is on the complementary strand.

<sup>b</sup> - In *C. coli* VC167 *pseA* (Cj1316c) is present but as a pseudogene.

<sup>c</sup> - All homopolymeric sequences are located within a gene unless stated. The figure in brackets indicates the consensus number of residues in that tract in the *C. jejuni* NCTC 11168 genome sequence (Parkhill *et al.*, 2000).

<sup>d</sup> - Presence of a non-consensus number of residues in the homopolymeric tracts between these coding sequences would allow them to be expressed as a single gene, as in Gundogdu *et al.* (2007)

<sup>1</sup> - Goon *et al.* (2003)

<sup>2</sup> - Guerry *et al.* (2006)

<sup>3</sup> - McNally *et al.* (2006a)

<sup>4</sup> - Thibault *et al.* (2001)

<sup>5</sup> - Chou *et al.* (2005)

<sup>6</sup> - Logan *et al.* (2002)

<sup>7</sup> - McNally *et al.* (2007)

<sup>8</sup> - van Alphen *et al.* (2008)

<sup>9</sup> - Schirm *et al.* (2003)

### 1.8.3 Mechanism of flagellin glycosylation

Whereas the *N*-linked glycosylation system of *Campylobacter* glycosylates a relatively wide range of proteins with a motif, Asn-Xaa-Ser/Thr, linked to the transfer of glycans, the *O*-linked system appears to be more specific as only the flagellin structural proteins are known to be glycosylated and there is no apparent motif linked to glycosylation. Other prokaryotic glycosylation systems have been associated with the cell membrane in the periplasm (Aas *et al.*, 2007, Chaban *et al.*, 2006 and Smedley *et al.*, 2005); this can not be true for flagellin glycosylation as flagellin monomers are secreted through the flagellar apparatus to the tip of the growing filament and never enter the periplasm. It is possible that glycosylation takes place in the cytoplasm, possibly in association with the flagellar basal body, although there is no evidence to support this; alternatively it may be at the level of transit across the cytoplasmic membrane. As there is no motif linked to glycosylation, as there is with the *N*-linked system, it is not easy to identify the mechanism that determines which serine and threonine residues are to be glycosylated. It is possible that, as the glycosylations are localised to the central domains of the flagellin, folding of the protein plays a part, although it is also possible that glycosylation in the central domain is determined by function only.

### 1.8.4 Biological role of *O*-linked glycosylation in *Campylobacter*

The *Campylobacter* flagellin glycosylation locus is, as previously mentioned, highly variable, containing many genes which encode enzymes involved in the biosynthesis of a variety of complex carbohydrates and have the potential to be phase variable by means of slipped-strand mispairing of homopolymeric runs of single nucleotides. The potential for generating so wide a diversity of flagellin glycoproteins

suggests a mechanism for immune system or bacteriophage evasion by antigenic diversity; this is supported by the fact that the flagellin is both surface exposed and an immunodominant protein. However, it has been shown by Schirm *et al.*, (2003) that the membrane sheathed (hence not exposed) flagella of *H. pylori* are also glycosylated with pseudaminic acid, which could suggest a role other than immune system evasion for flagellin glycosylation. This role may be in flagellar assembly and/or function. An increasing body of evidence suggests that glycosylation is required for the assembly and/or function of *Campylobacter* flagella. Upon mutagenesis several genes located in the flagellin modification locus give a non-motile phenotype (see Table 1.2). A mutant in *pseB*, which encodes the first stage of the PseAc synthesis pathway, in *C. jejuni* 81-176 is unable to synthesis PseAc, lacks flagella and accumulates unglycosylated flagellin intracellularly (Goon *et al.*, 2003). It is also possible that flagellar glycans contribute to flagellin subunit-subunit interactions in compensation for those mutations which allow for evasion of TLR5 by *Campylobacter* flagellin but also affect flagellin subunit interactions in flagellar biogenesis.

It is not currently evident how flagellin glycosylation is involved in flagellar assembly; it is quite possible that the effects on flagellar assembly may be incidental, and glycosylation may have a different and as yet unclear primary role. It is likely to be of some importance, as a relatively large part of the compact genome of *C. jejuni* appears to be devoted to these processes. Likewise almost nothing is understood about the regulation of flagellin glycosylation in *Campylobacter* and how it is integrated with other regulatory circuits.

### 1.8.5 Regulation of flagellin glycosylation genes by NssR

The response to nitric oxide (NO) related stress in *C. jejuni* is regulated by NssR (Cj0466), a member of the FNR-CRP family of transcriptional regulators (Elvers *et al.*, 2005); more details are given in Section 1.3.6. NssR controls the expression of a regulon of at least four genes in response to nitrosative stress in both the *C. jejuni* NCTC 11168 sequence strain and its ‘original’ motile variant, detailed in section 1.6.2 and here designated 11168-O WT and 11168-GS WT respectively, representing original and genome sequenced.

It has been observed (Sue Turner, unpublished data) that in the ‘original’ strain background, 11168-O, a deletion mutant in *nssR*, designated 11168-O 0466<sup>-</sup>, exhibited lower levels of expression of several genes in the flagellin glycosylation region than was observed the wild-type, under normal, NO-free, conditions. Microarray analysis was carried out for both the wild-type and NssR mutant (11168-O WT 0466<sup>-</sup>) without nitrosative stress, and 17 genes located in the flagellin glycosylation region were found to be down-regulated in the 11168-O 0466<sup>-</sup> mutant in comparison to the wild-type. These genes are located between the direct repeats Cj1318 and Ci1336 and have been designated the flagellin modification region (FMR) in this study. This NO-independent difference in gene expression suggests that NssR is involved, either directly or indirectly, in the regulation of this cluster of genes. Examination of motility in the 11168-O 0466<sup>-</sup> mutant indicated that although it is still motile, it is less motile than the parent strain. Differences in mass of the flagellin protein were also observed by SDS-PAGE. An NssR mutant was also constructed in the 11168-GS strain background and microarray analysis conducted, without NO stress, for the 11168-GS wild-type and mutant. The 17 genes were found not to be down-regulated in the 11168-GS strain (Lynne Lawrence, unpublished data), suggesting that expression differences in this

region may contribute to the differences observed between the 11168-O WT and 11168-GS WT strains.

Amongst the cluster of 17 genes down regulated in the ‘original’ background *nssR* mutant, there are several that are known to be involved in the post-translational glycosylation of flagellin in other *Campylobacter* strains, such as *ptmA-G* and *pseD*. How NssR is involved in the regulation of these genes is unknown, as are the effects of the down-regulation of these genes themselves.

## 1.9 Aims

Whilst the nature of the glycosyl groups present on *Campylobacter* flagellins, the genes responsible and even, to some degree, the functions of flagellin glycosylation are being discovered, no information is available on the regulation of flagellin glycosylation in *Campylobacter*. As a relatively large number of genes, given the small size of the *Campylobacter* genome, are involved in flagellin glycosylation, regulation of this process would appear to be important. The first aim of this project is to investigate the detailed response and potentially the mechanism by which NssR regulates the genes of the FMR, to attempt to establish whether NssR acts directly on the genes themselves or indirectly, via another regulator.

Motility is vital for *Campylobacter* virulence; several genes involved in flagellar glycosylation, when mutated, cause a loss or alteration of motility. The NssR mutant created in the original background appears significantly less motile than its parent strain, although it retains a certain level of motility; the 11168-GS background strains are non-motile. The motility of the *Campylobacter* strains used in this study will be examined and recorded using rigorous and potentially quantitative methods.



The glycosyl groups present on the flagellins of two *Campylobacter* strains (*C. jejuni* 81-176 and *C. coli* VC167) have been deduced and located to specific serine and threonine residues. Whilst the glycosyl modifications present on *C. jejuni* 11168 flagellin have been identified, the particular residues upon which they reside have not. The final aim of this project is to identify and locate, using mass spectrometry, the glycosyl residues present on the flagellin of the *Campylobacter* strains used here.

Taken together, data gained using these approaches will significantly enhance knowledge and understanding of the mechanisms and role of flagellin glycosylation in *C. jejuni* NCTC 11168.

# Chapter II: General Materials and Methods

## 2.1 Bacterial culture media

All culture media were obtained from Oxoid (Basingstoke, UK), prepared using distilled water and sterilised by autoclaving at 121°C at 15 p.s.i for 15 minutes.

Supplements were added to the media after it had cooled to 52°C; all media were stored at 4°C.

### Muller Hinton Broth (CM0405)

Dehydrated Beef Infusion	From 300 g
Casein hydrolysate	17.5 g
Starch	1.5 g

Made up to 1 litre with distilled water

For 1 litre Muller Hinton agar add 17 g agar

### Luria Bertani Broth

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g

Made up to 1 litre with distilled water

For 1 litre Luria Bertani agar add 17 g agar

## 2.2 Antibiotics and other additives

All antibiotics and additives used in this study are listed in Tables 2.1 and 2.2 respectively. All antibiotics and additives dissolved in water were filter-sterilised with 0.22  $\mu\text{m}$  syringe filters (Pall Corporation, Portsmouth, UK) before use and stored in aliquots at  $-20^{\circ}\text{C}$ .

**Table 2.1 List of antibiotics**

Antibiotic	Stock solution ( $\text{mg.ml}^{-1}$ )	Concentration used ( $\mu\text{g.ml}^{-1}$ )
Ampicillin (Sigma, Gillingham, UK)	100 in $\text{H}_2\text{O}$	100
Chloramphenicol (Sigma, Gillingham, UK)	50 in $\text{H}_2\text{O}$	50
Kanamycin (Sigma, Gillingham, UK)	20 in absolute ethanol	20

**Table 2.2 List of additives**

Additive	Stock solution	Concentration used
Isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG, Bioline, London, UK)	0.1 M in $\text{H}_2\text{O}$	0.1 mM
5-Bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside (X-gal, Bioline, London, UK)	40 $\text{mg.ml}^{-1}$ in dimethyl formamide (Fluka, Gillingham, UK)	40 $\mu\text{g.ml}^{-1}$

## 2.3 Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are detailed in Table 2.3

**Table 2.3. Bacterial strains and plasmids used in this study.**

Bacterial strains and plasmids	Characteristics	Source or reference
<i>C. jejuni</i> NCTC 11168	The genome sequenced strain, here designated 11168-GS.	National collection of type cultures (HPA Culture Collections, Salisbury, UK)
<i>C. jejuni</i> 11168-GS 0466 <sup>-</sup>	11168-GS <i>nssR</i> ::Kan <sup>r</sup> , a Kan <sup>r</sup> cassette is inserted into the remains of the <i>nssR</i> gene, which has been deleted.	Strain made by Sue Turner at the University of Birmingham according to the method of Elvers <i>et al.</i> (2005)
<i>C. jejuni</i> 11168-GS 0466C	11168-GS 0466 <sup>-</sup> with the mutation complemented by insertion of <i>nssR</i> ::Cm <sup>r</sup> into Cj0752	Strain made by Sue Turner at the University of Birmingham according to the method of Elvers <i>et al.</i> (2005)
<i>C. jejuni</i> 11168-O	A version of the original clinical isolate, here designated 11168-O.	Gaynor <i>et al.</i> (2004)
<i>C. jejuni</i> 11168-O 0466 <sup>-</sup>	11168-O <i>nssR</i> ::Kan <sup>r</sup> , the same mutation as in 11168-GS but in the original stain background.	Elvers <i>et al.</i> (2005)
<i>C. jejuni</i> 11168-O 0466C	11168-O 0466 <sup>-</sup> with the mutation complemented by insertion of <i>nssR</i> ::Cm <sup>r</sup> into Cj0752	Elvers <i>et al.</i> (2005)
<i>E. coli</i> DH5α	F <sup>-</sup> Φ80dLacZ ΔM15. A standard strain used for cloning.	Invitrogen (Paisley, UK)
<i>E. coli</i> BL21 (DE3)	F <sup>-</sup> <i>ompT hsdS<sub>B</sub>(r<sub>B</sub><sup>-</sup>m<sub>B</sub><sup>-</sup>) gal dcm</i> (DE3). A standard strain for protein expression.	Bioline (London, UK)
pET 22b	Expression vector, amp <sup>r</sup> , with C-terminal 6xHis•Tag periplasmic export sequence to aid recovery of recombinant proteins	Novagen (Darmstadt, Germany)

pDEX_Fcc	Contains 3x FLAG sequence, kan <sup>r</sup> , contains two multiple cloning sites allowing insertion of sequences for homologous recombination onto the <i>Campylobacter</i> genome	For sequence see appendix 1.
pGEM-T Easy	High copy number cloning vector, amp <sup>r</sup> , allows blue/white screening by insertional inactivation of $\beta$ -galactosidase $\alpha$ -peptide	Promega (Madison, USA)

## 2.4 Culture conditions for *E. coli* and *C. jejuni*

*Campylobacter jejuni* strains were routinely grown from frozen on Muller Hinton (MH) agar (Oxoid, Basingstoke, UK) at 37°C in a microaerobic atmosphere containing 5% (v/v) O<sub>2</sub>, 10% (v/v) CO<sub>2</sub> and 85% (v/v) N<sub>2</sub>, generated by a MACS 500 variable atmosphere incubator (Don Whitley Scientific Ltd, Shipley, UK) Liquid cultures were grown in MH broth in vented cap tissue culture flasks (Becton Dickinson, Oxford, UK) at 37°C under the same microaerobic conditions, with shaking at  $\approx$ 220 rpm.

*Escherichia coli* strains were grown on Luria Bertani (LB) agar medium at 37°C under aerobic conditions, liquid cultures in LB broth were incubated aerobically with shaking at  $\approx$ 100rpm.

## 2.5 Storage of *E. coli* and *C. jejuni*

Strains were stored at -80°C in either MH broth (*C. jejuni*) or LB broth (*E. coli*) containing 15% (v/v) glycerol (Fisher Scientific, Loughborough, UK) and 2% (v/v) foetal bovine serum. Strains were routinely recovered from stocks.

## 2.6 Isolation of genomic DNA

Cells were grown overnight in broth culture using the strain specific culture conditions given above. To produce a pellet, 40 ml of *E. coli* culture or 60 ml of *C. jejuni* culture was centrifuged for 10 min at 5,000 x g and the supernatant discarded. Genomic DNA was then isolated using the Qiagen genomic DNA extraction kit (Qiagen, Crawley, UK), with a 500/G tip, according to the manufacturers instructions. The bacterial pellet was re-suspended in 11 ml buffer B1, with 22 µl RNase (100 mg.ml<sup>-1</sup>), by vortexing to form a homogeneous suspension. To lyse cells 300 µl lysozyme (100 mg.ml<sup>-1</sup>) in TE buffer and 500 µl of Qiagen proteinase K (Qiagen, Crawley, UK) were added to the solution, which was then incubated at 37°C for 90 minutes. Following incubation, 4 ml buffer B2 was added to the sample, mixed by inverting the tube several times and incubated at 50°C for 60 minutes. DNA was purified using the Qiagen genomic tip 500/G (Qiagen, Crawley, UK). The genomic tip was equilibrated with 10 ml buffer QBT, the sample was then applied to the genomic tip and allowed to empty by gravity flow. The tip was washed twice with 15 ml buffer QC and DNA eluted into a clean collection tube with 15 ml buffer QF which had been warmed to 50°C to increase DNA yield. DNA was precipitated with 0.7 volumes of room temperature isopropanol and spooled using a glass rod. The DNA was then transferred to an Eppendorf tube containing 1 ml TE buffer and allowed to dissolve on an orbital shaker at 4°C overnight. To determine DNA yield and quality 5 µl of DNA in the following dilutions: neat, 1:10, 1:20, 1:50, 1:100, together with molecular size marker (Hyperladder I, Bioline, London, UK) was examined by agarose gel electrophoresis. DNA concentration and quality was also examined using a Nanodrop spectrophotometer.

## 2.7 Isolation of plasmid DNA

### 2.7.1 Qiagen Midi Kit

Plasmid DNA was extracted using the Qiagen Midi Kit (Qiagen, Crawley, UK), according to the manufacturers' instructions, when a high yield of concentrated plasmid DNA was required, for example for electroporation. A 10 ml liquid culture of *E. coli* was grown to an OD<sub>600nm</sub> of 0.6; 2 ml of this culture was used to inoculate 25 ml of fresh media, which was then grown overnight. Bacteria were then harvested by centrifugation at 6,000 x g for 10 minutes at 4°C. The pellet was resuspended in 4 ml buffer P1 containing RNase A at a final concentration of 100 µg.ml<sup>-1</sup> and vortexed to ensure a homogenous suspension. Buffer P2 (4 ml) was added, mixed by inversion and incubated for 5 minutes at room temperature. Chilled buffer P3 (4 ml) was added, mixed by inversion and incubated for 15 minutes on ice. The sample was then centrifuged at 15,000 x g for 30 minutes at 4°C, the supernatant was removed promptly after centrifugation was complete, retained and centrifuged again at 15,000 x g for 15 minutes at 4°C.

A QIAGEN-tip 100 was equilibrated with 4 ml Buffer QBT and allowed to empty by gravity flow. The supernatant from the previous step was applied to the column and allowed to enter the resin by gravity flow. The column was washed with 2 x 10 ml aliquots of buffer QC; the DNA was then eluted in 5 ml buffer QF. DNA was precipitated by adding 3.5 ml (0.7 volumes) of room-temperature isopropanol, mixing and centrifuging immediately at 15,500 x g for 30 minutes at 4°C. The supernatant was decanted and the DNA pellet washed twice with 2 ml room-temperature 70% ethanol with centrifugation at 15,500 x g. After removal of the supernatant, the pellet was air-dried for 10 minutes at room-temperature and redissolved in 1 ml TE buffer (pH 8.0).

Yield was determined a Nanodrop spectrophotometer and purity examined by agarose gel electrophoresis as required.

### 2.7.2 QIAprep Miniprep

The QIAprep Miniprep (Qiagen, Crawley, UK) was used according to the manufacturers' instructions as a method of preparing relatively small amounts of plasmid DNA, usually for PCR screens or DNA sequencing. Cultures of *E. coli* were grown overnight in 5 ml LB broth, supplemented with the appropriate antibiotic, which was inoculated with a single colony from an LB agar plate. Bacterial cells were harvested with centrifugation at 8,000 x g for 10 minutes at 4°C. The pelleted cells were resuspended in 250 µl buffer P1, transferred to a 1.5 ml Eppendorf tube, where 250 µl buffer P2 was added, and the tube inverted to mix. Buffer N3 (350 µl) was added, immediately mixed and centrifuged at 13,000 x g for 10 minutes. The supernatant was applied to a QIAprep spin column which was then centrifuged for 60s at 13,000 x g and the flow-through discarded. The column was washed with 0.5 ml buffer PB and 0.75 ml buffer PE, the flow-through was discarded and the column centrifuged for an additional 1 minute to remove all traces of the wash buffers. The column was transferred to a clean 1.5 ml Eppendorf tube and plasmid DNA eluted with 50 µl buffer EB. DNA yield and purity was assessed using a Nanodrop spectrophotometer or agarose gel electrophoresis as necessary.



## 2.8 Agarose gel electrophoresis

DNA was examined and purified on a 0.7% w/v agarose (Bioline, London, UK) gel in 0.5% Tris-borate EDTA (TBE) buffer. To visualise DNA, 0.5 $\mu$ g.ml<sup>-1</sup> ethidium bromide (Fisher, Loughborough, UK) was mixed into the gel whilst still molten. Prior to loading the sample was mixed with 5x loading buffer (Bioline, London, UK), 5-20  $\mu$ l of sample was run as required. Hyperladder I (Bioline, London, UK; 200bp- 10kb) or Hyperladder IV (Bioline, London, UK; 100bp- 1kb) was used as a molecular weight marker. The gel was usually run at 80V for 1.5h in 1 x TBE buffer.

### 5 x TBE buffer

Tris-HCl (Fisher, Loughborough, UK)	54 g
Boric acid (Fisher, Loughborough, UK)	27.5 g
0.5 M EDTA (Fisher, Loughborough, UK)	20 ml
Sterile distilled H <sub>2</sub> O	to 1L

## 2.9 Polymerase chain reaction (PCR)

### 2.9.1 Primer design

Primers for PCR were designed based on the following criteria: a melting temperature ( $T_m$ , °C) calculated as 4°C x (G + C) + 2°C x (A +T) of as close as possible to 60°C, with each primer pair within 5°C of each other where possible. A primer length of 20-30 nucleotides, with as near as possible to a 50 % GC content was preferred, although this was not always achievable as *Campylobacter* has a very AT-rich genome. Where possible several G or C residues were included at the 3' end of the

primers (3' clamp), to provide a stronger annealing region from which replication can be initiated, again this was not always possible. Primer pairs were chosen to avoid cross complementarity and inverted repeat sequences which could lead to secondary structure formation. CampyDB pattern search (<http://campy.bham.ac.uk>) was used to ensure that each primer sequence was unique to one location within the genome. Primers to be used for cloning were designed with the necessary restriction sites at their 5' ends. Primers were obtained as lyophilized DNA from Eurofins MWG Operon (Ebersberg, Germany) and resuspended Milli-Q purified water (Millipore, Billerica, USA) pure water to a final concentration of 100µM.

### 2.9.2 PCR

For PCR reactions where the amplicon was less than 3 kb in length, either BioMix Red (Bioline, London, UK) or GoTaq Flexi DNA Polymerase (Promega, Madison, USA) was used; GoTaq was used exclusively in place of BioMix Red after problems with the efficiency of the later were encountered. For PCR reactions greater than 3 kb in length, or for those reactions where high fidelity was required, for example for DNA to be used in cloning reactions, BIO-X-ACT (Bioline, London, UK) was used as, it possesses higher fidelity than *Taq* due to an improved 3'-5' exonuclease proof reading activity. Reactions were carried out in 0.2 ml PCR tubes (Star Lab, Milton Keynes, UK), with 50µl of reaction solution. To PCR screen clones, the supernatant of colony material, taken from cells added to 100 µl of sterile distilled water and boiled for 10 minutes and centrifuged, was used as a template; for cloning purposes genomic DNA was always used as a template.

The following components were used for reactions with BioMix Red:

2 x BioMix Red	25.0 µl
Forward primer (100 µM)	2.0 µl
Reverse Primer (100 µM)	2.0 µl
Template (genomic DNA)	2.0 µl
Sterile distilled water to	50.0 µl

For reactions using either GoTaq or BIO-X-ACT long the following components were used:

5 x Reaction buffer	10.0 µl
dNTP mix (25 mM each)	0.5 µl
MgCl <sub>2</sub> (100 mM)	4.0 µl
Forward primer (100 µM)	1.0 µl
Reverse primer (100 µM)	1.0 µl
DNA polymerase - BIO-X-ACT (100U.µl)	1.0 µl
- GoTaq (200U.µl)	0.25 µl
Sterile distilled water	to 50 µl

The following protocol was used, unless otherwise stated:

1. Heating at 94°C for 2 minutes
2. 15 x cycles
  1. Denaturation at 94°C for 30s
  2. Annealing at  $T_m$  of the annealing part of primers only for 30s
  3. Extension at 72°C for estimated time ( $\approx$ 1 minute per kb)
3. 15 x cycles
  1. Denaturation at 94°C for 30s
  2. Annealing at  $T_m$  of whole primers (including restriction sites) for 30s
  3. Extension at 72°C for estimated time ( $\approx$ 1 minute per kb)
4. Final extension at 72°C for 10 minutes

After PCR, DNA was purified using either a QIAquick PCR purification kit (Qiagen, Crawley, UK) or a QIAquick gel extraction kit (Qiagen, Crawley, UK) if the desired DNA was contaminated with other DNA products. See section 2.10

## 2.10 DNA purification

DNA purification was carried out as required, using either a QIAquick PCR Purification Kit or, if the required piece of DNA was contaminated with other DNA, by subjecting the sample to agarose gel electrophoresis, excising the required band and purifying it using a QIAquick Gel Extraction Kit (Qiagen, Crawley, UK).

### 2.10.1 DNA purification by QIAquick Purification Kit

A QIAquick PCR Purification Kit (Qiagen, Crawley, UK) was used according to the manufacturers' instructions to clean DNA. Buffer PB (5 volumes) was added to 1

volume of PCR product, mixed by pipetting and added to a QIAquick spin column in a 2 ml collection tube. The spin column was centrifuged for 1 minute at 8,000 x g, the flow-through discarded and the column washed with 750µl buffer PE. The column was then centrifuged for an additional 1 minute, to remove all traces of wash buffer, and placed into a clean 1.5 ml Eppendorf tube. Buffer EB (50 µl) was added to the spin column membrane and the sample eluted. DNA yield and quality was checked using a Nanodrop spectrophotometer or agarose gel electrophoresis as required.

### **2.10.2 DNA purification by QIAquick Gel Extraction Kit.**

The DNA fragment to be purified was run out on a 0.7% agarose gel until clearly separated from any contaminating DNA, the required band was then excised from the gel using a sharp, clean scalpel blade, taking care to remove as much unnecessary agarose as possible. The mass of the slice was determined and 3 volumes of buffer QG added. The slice was then incubated at 50°C with vortexing every 2 minutes until completely dissolved. Once dissolved, 1 volume of isopropanol was added, mixed by pipetting and the sample added to a QIAquick spin column in a 2 ml collection tube. The spin column was centrifuged at 13,000 x g for 1 minute, the flow-through discarded and the column washed with 500µl buffer QC and 750µl buffer PE. The column was then centrifuged for an additional minute to remove any traces of wash buffer remaining, placed in a clean 1.5 ml Eppendorf tube and the DNA eluted with 50 µl buffer EB. DNA yield and quality was checked using a Nanodrop spectrophotometer or by agarose gel electrophoresis as required.

## 2.11 Restriction digestion

DNA was digested as needed with the required restriction enzyme, obtained from New England Biolabs (NEB, Hitchin, UK). The digestion was usually carried out in a 50  $\mu$ l final volume containing 5  $\mu$ l of the appropriate 10 x NEBuffer, DNA and 1  $\mu$ l of enzyme per  $\mu$ g of DNA, to a maximum of 3  $\mu$ l per reaction. The digestion was incubated for 1h usually at a temperature of 37°C, unless a specific temperature was required by the enzyme. Where digestion with two restriction enzymes was required, the buffer with the best suitability for both was used, in cases where this was impossible, DNA was digested with the first enzyme and the DNA cleaned prior to digestion with the second enzyme. DNA was cleaned with either a QIAquick PCR purification kit or a QIAquick gel extraction kit as required.

## 2.12 Dephosphorylation of vector DNA

Plasmid cloning vectors were dephosphorylated after digestion with a single restriction enzyme to decrease the chance of self-ligation occurring. Calf intestinal alkaline phosphatase (CIP), obtained from NEB (Hitchin, UK), was added directly to the restriction digestion reaction in the ratio of 0.5 units of CIP per  $\mu$ g of vector and incubated at 37°C for 1h. DNA was then cleaned with a QIAquick gel extraction kit (Qiagen, Crawley, UK).

## 2.13 Ligation

To clone a piece of DNA, both insert and vector were digested with the same restriction enzymes then ligated together. T4 DNA ligase (NEB, Hitchin, UK) was used in a 20  $\mu$ l reaction volume containing 2  $\mu$ l 10 x ligase buffer, vector DNA, insert DNA,

1 µl T4 DNA ligase and sterile distilled water. The reaction was incubated at room-temperature for 20 minutes. A control reaction, where the insert DNA was substituted for an equal volume of sterile distilled water, was used to assess the amount of re-ligation of vector occurring. Insert and vector DNA was added in the ratio 3:1, the following equation was used to calculate the amount of insert DNA required:

$$\frac{\text{Vector DNA (ng)} \times \text{Size of insert (kb)}}{\text{Size of vector (kb)}} \times \text{molar ratio of} \frac{\text{Insert}}{\text{Vector}} = \text{Insert DNA (ng)}$$

## 2.14 Preparation of chemically competent *E. coli* cells

Chemically competent *E. coli* were produced using the calcium chloride method. A 50 µl aliquot of an overnight culture of *E. coli* was inoculated into 5 ml LB broth and grown at 37°C until the OD<sub>600nm</sub> reached 0.3. The cells were then centrifuged at 10,000 x g for 3 minutes, the supernatant removed, the pellet resuspended in 1 ml 0.1M CaCl<sub>2</sub> and left on ice for 30 minutes. The cells were then washed twice in 0.1M CaCl<sub>2</sub> and left on ice for 1h after the final wash. Finally the cell suspension was dispensed into 100 µl aliquots, flash frozen in liquid nitrogen and stored at -80°C until required.

## 2.15 Transformation

As required 100 µl aliquots of chemically prepared competent *E. coli* were taken from storage at -80°C, kept on ice for approximately 20 minutes until thawed and then mixed by flicking the tube. DNA from a ligation reaction, in a total volume of no greater than 10 µl, was added to the cells and mixed by flicking. The cells were then incubated on ice for 10 minutes and heat shocked at exactly 42°C for 50s, before being

placed back onto ice for 2 minutes. Ice-cold SOC medium (900 µl) was added to the cells, which were then incubated aerobically at 37°C for 1h without shaking. Cells were then spread onto LB agar plates containing the necessary antibiotics and supplements. A positive control consisting of the high copy number plasmid pUC19 was used to assess the competency of the cells. A ‘cells only’ control, consisting of competent cells put through the transformation procedure without the addition of DNA, was used to assess the antibiotic sensitivity of the cells if spread onto supplemented plates and to assess the viability of the cells following the procedure, when spread onto plain LB agar. The ligation control detailed in section 2.13 was also transformed to assess the amount of circular vector DNA present due to re-ligation of the vector occurring in the reaction or failure to achieve complete digestion.

#### SOC medium

Tryptone	2.0 g	MgCl <sub>2</sub> (1M)	0.5 ml
Yeast extract	0.5 g	MgSO <sub>4</sub> (1M)	0.5 ml
NaCl (1M)	1.0 ml	Glucose (2M)	1.0 ml
KCl (1M)	0.25 ml	Sterile distilled water	to 100 ml

### **2.16 Preparation of electrocompetent *C. jejuni* cells**

To prepare electrocompetent *C. jejuni*, cells were grown on a MH agar plate as a lawn and harvested in 2 ml MH broth with a sterile loop. After centrifugation at 10,000 x g for 20 minutes at 4°C the cells were gently resuspended in 1 ml ice-cold wash buffer. Centrifugation and washing were repeated three times, the cells were then



resuspended in 1 ml wash buffer, dispensed into 100 µl aliquots, snap frozen in liquid nitrogen and stored at -80°C until required.

#### Wash buffer

Sucrose	9.31 g
Glycerol	1.5 ml
Sterile distilled water	to 100 ml

## **2.17 Electroporation**

When required, 100 µl aliquots of electrocompetent *C. jejuni* were taken from storage at -80°C and kept on ice for approximately 20 minutes until thawed. Between 1-3 µg DNA, in volume no greater than 2 µl, was added to pre-cooled cuvettes and kept on ice. Competent cells (100 µl) were added to each cuvette, mixed and electroporated at 2.5kV, 200 Ω and 25 µF using a BioRad (Hemel Hempstead, UK) gene-pulser with a time constant between 4-6 ms. Cuvettes were flushed with 100 µl SOC buffer (section 2.15), the cells spread onto non-selective MH plates and incubated overnight to allow time for the expression of antibiotic resistance genes to occur. After overnight incubation (preferably lasting no longer than 20h) cells were harvested with 1 ml MH broth and spread onto MH agar plates containing a reduced concentration (40%) of the appropriate antibiotic(s). These plates were incubated for up to five days and any resulting colonies purified on MH agar plates containing the full concentration of antibiotic(s).

## 2.18 DNA sequencing

DNA was sequenced on an automated ABI 3700 sequencer using the ‘ABI PRISM BigDye Terminator Cycle Ready Kit’ (Applied Biosystems, Foster City, USA), at the Genomics Laboratory of the School of Biosciences, University of Birmingham. The primers used were either the primers used for PCR or specifically designed for sequencing, depending upon the type of sequencing to be carried out; in all cases a final primer concentration of 3.2 pM was used. The amount of DNA added to the reaction was dependant upon the size of the DNA fragment to be sequenced, Table 2.4 details the amount of DNA required; the total reaction volume was 10 µl, sterile distilled water was added to the reaction if necessary.

**Table 2.4. Amounts of DNA required for sequencing**

DNA		Amount
PCR product, size:	100 - 200 bp	1 - 3 ng
	200 - 500 bp	3 – 10 ng
	500 - 1000 bp	5 - 20 ng
	1000 - 2000 bp	10 - 40 ng
	>2000 bp	40 ng
Double stranded DNA		200 - 500 ng
Genomic DNA		2 - 3 µg

## **2.19 Transmission Electron Microscopy (EM)**

The sample to be examined was suspended in a 1% (v/v) ammonium molybdate solution containing 70  $\mu\text{g}.\text{ml}^{-1}$  bacitracin; for samples containing bacteria, a slightly turbid suspension was desirable. A 10  $\mu\text{l}$  drop of the sample suspension was placed into a sterile Petri dish and a Formvar<sup>TM</sup> carbon coated grid inverted and applied to the sample for 2 minutes. Grids were blotted dry, taking care not to dislodge the sample, and viewed on a JEOL JEM 1200 EX transmission electron microscope.

## **2.20 Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

SDS-PAGE was performed by the method of Laemmli (1970), using a MiniProtean-3 mini-slab gel apparatus (BioRad Laboratories, Hemel Hempstead, UK) according to the manufacturers instructions, the buffers and solutions used here are detailed in Appendix 2. Glass plates, spacers and combs were washed in water and polished with ethanol prior to assembly. 12.5% acrylamide resolving gels and 4% acrylamide stacking gels were used. Samples to be analysed were added to sample buffer in the ratio 2:1 and heated to 100°C for 10 minutes. Electrophoresis was carried out for 2h at 100V in 1 x electrode buffer; both Pre-stained broad range Protein Markers (NEB, Hitchen, UK) and unstained Perfect Protein Markers (Novagen, Darmstadt, Germany) were run with the protein sample. Gels were stained on a shaker using Coomassie blue staining solution for 1h and de-stained using destain solution, which was changed every 30 minutes for 3h. Gels were recorded by scanning with a BioRad (Hemel Hempstead, UK) GS-710 Densitometer.

# Chapter III: Transcriptional analysis of the flagellin modification region of *C. jejuni* 11168

## 3.1 Introduction

Flagellin glycosylation has been studied most extensively in two particular *Campylobacter* strains, *C. jejuni* 81-176 and *C. coli* VC167. Studies such as those carried out by McNally *et al.* (2007) and Thibault *et al.* (2001) have led to the characterisation of the flagellar glycans present in both species. Other work has focused on the assignment of functions to many flagellar glycosylation genes and to elucidation of the biochemical pathways by which flagellar glycans are synthesised (Guerry *et al.*, 2006; McNally *et al.*, 2006a; Obhi and Creuzenet, 2005). Some work has been carried out in *C. jejuni* 11168, particularly by Logan *et al.* (2009), who identified the presence of several flagellar glycans including pseudaminic acid itself and the derivative PseAm, as well as the two legionaminic acid derivatives previously identified on *C. coli* VC167 flagellin. Given the homology between many flagellin glycosylation genes found in *C. jejuni* 11168, *C. jejuni* 81-176 and *C. coli* VC167 it is perhaps not surprising that many similar glycans should also be present on the flagellin of these strains. However, the flagellin glycosylation locus in *C. jejuni* 11168 is larger than any yet identified in *Campylobacter*, so it could also be expected that there would be other novel glycans present on the flagellin of *C. jejuni* 11168 (Guerry *et al.*, 2006; Parkhill *et al.*, 2000). The study by Logan *et al.* (2009) also identified two previously unknown pseudaminic

acid related glycans, both dimethylglyceric derivatives of both pseudaminic acid and acetamidino pseudaminic acid; these two new glycans were together the most prevalent modifications found on *C. jejuni* 11168 flagellin.

There is relatively little known about the regulation of flagellin glycosylation. Goon *et al.* (2003) identified that *pseB* (Cj1293), which forms the initial step in pseudaminic acid synthesis, was expressed from both  $\sigma^{54}$  and  $\sigma^{70}$  promoters in *C. jejuni* 81-176. *pseB* was found to be transcribed alone by  $\sigma^{54}$ , which is involved in control of the mid-stage flagellar regulon, and co-transcribed with the preceding gene Cj1292 under the control of  $\sigma^{70}$ . *pseB* is a dehydratase and mediates the first stage in the biosynthesis of the major flagellar glycan, pseudaminic acid. Thus, if flagellin glycosylation has a role in flagellar biogenesis, it would be necessary for those genes involved in glycan biosynthesis and transfer to be regulated temporally with the late-stage flagellar structural genes, such as the late basal body proteins and the flagellin and hook proteins, so that the flagellin glycosylation machinery was already in place when the flagellin, which is consistent with  $\sigma^{54}$  activity (Carrillo *et al.*, 2004; Wosten *et al.*, 2004). Co-regulation by  $\sigma^{70}$  may ensure that there is a certain level of *pseB* present in the cell at all times in case of a sudden need to synthesise flagellar glycans, as *Campylobacter* flagella are subject to phase variation. There is also a certain level of cross-talk between the *O*-linked and *N*-linked pathways via PseB, which has homology to the *N*-linked dehydratase PglF (McNally *et al.*, 2006b; Schoenhofen *et al.*, 2006) and it is possible that the  $\sigma^{70}$  controlled expression of *pseB* is linked somehow to a function within the *N*-linked pathway, although this remains unclear. Carrillo *et al.* (2004) also identified several genes involved in flagellin glycosylation that were located downstream of putative  $\sigma^{28}$  promoters, including the legionaminic biosynthesis genes *ptmA* and *ptmB* and pseudaminic acid biosynthesis genes *pseA*, *pseG* and *pseH*. The

potential co-regulation of flagellin glycosylation genes by the two major flagellar regulators,  $\sigma^{28}$  and  $\sigma^{54}$ , helps to support the idea of flagellin glycosylation having an integral role in flagellar function; some evidence for which has already been provided in the form of mutants studies by Karlyshev *et al.* (2002) and Goon *et al.* (2003).

Although, as detailed above, there is some evidence of the mechanism(s) of regulation of certain genes, for the majority of flagellin glycosylation genes it is entirely unclear; there is no evidence of regulation of any flagellin glycosylation associated genes by anything other than the flagellar associated sigma-factors  $\sigma^{54}$  and  $\sigma^{28}$ .

The overall aim of this study is to attempt to determine the effects that NssR, the nitrosative stress response regulator in *Campylobacter*, has on a small subset of the flagellin glycosylation genes. An initial observation during the work carried out on NssR and its associated regulon by Elvers *et al.* (2005) indicated that an *nssR* deletion mutant in the 'original' strain background, 11168-O, exhibited lower levels of expression of a small number of genes in the flagellin glycosylation region than the wild-type, under normal, NO-free, conditions (Sue Turner, unpublished data). Subsequently, microarray analysis of the wild-type and *nssR* mutant (11168-O WT 0466<sup>-</sup>), without nitrosative stress, positively identified 17 genes located in the flagellin glycosylation region, which were down-regulated in the 11168-O 0466<sup>-</sup> mutant in comparison to the wild-type. These genes are located between the direct repeats Cj1318 and Cj1336 and have been designated the flagellin modification region (FMR) in this study. When an *nssR* mutant was constructed in the genome sequenced background (11168-GS) no differences in gene expression in this region were identified (Lynne Lawrence, unpublished data). The NO-independent difference in gene expression between the wild-type and  $\Delta nssR$  mutant in the original strain background suggests that NssR is involved, either directly or indirectly, in the regulation of this cluster of genes.

The fact that no gene expression differences were identified in the genome sequenced background suggests that overall expression differences in this region may contribute to the differences observed between the 11168-O WT and 11168-GS WT strains, including differences in motility and flagellin mass.

Examination of motility in the 11168-O 0466<sup>-</sup> mutant indicates that, although the strain is still motile, it is less motile than the parent strain; differences in mass of the flagellin protein were also observed by SDS-PAGE. Amongst the cluster of 17 genes down regulated in the ‘original’ background *nssR* mutant, several are known to be involved in the post-translational glycosylation of flagellin in other *Campylobacter* strains, such as *ptmA-G*, which are involved in legionaminic acid biosynthesis in *C. coli* VC167 and *pseD*, which is implicated in the attachment of acetamidino pseudaminic acid to flagellin monomers in *C. jejuni* 81-176 (Guerry *et al.*, 2006).

The promoter regions of those genes regulated by Crp-Fnr superfamily members contain distinctive recognition motifs, such as the inverted repeat sequence TTGAT-N<sub>4</sub>-ATCAA recognised by FNR proteins (Eiglmeier *et al.*, 1989). Elvers *et al.* (2005) discovered a consensus sequence, TTAAC-N<sub>4</sub>-GTAA, upstream of all of the NssR-dependent genes that they identified, which is therefore predicted to be the NssR-binding motif, at least under conditions of nitrosative stress. How NssR is involved in the regulation of these genes is unknown, as are the effects of the down-regulation of these genes themselves, other than that a *ΔnssR* mutant is defective in motility and has a lower flagellin mass than the wild-type.

## Aims

This part of the study aims to examine gene expression in the 17 gene region down-regulated in the 11168-O background *nssR* mutant. Using transcript mapping by conventional qualitative RT-PCR and assays of gene expression by real-time quantitative PCR (qPCR), transcriptional organisation of the locus will be assessed in detail. The upstream regions of Cj1318 (containing a homopolymeric sequence), Cj1319 and Cj0466 will also be examined for the presence of sequence changes in these areas that could explain the phenotypic differences seen between the strains; inversion of the DNA between the direct repeats Cj1318 and Cj1336 will also be tested for.

Chromatin immunoprecipitation coupled with high density microarrays, also known as ChIP-chip, will be attempted to try to identify locations at which NssR binds across the 11168-O WT genome. This will allow identification of any other genes regulated by NssR under normal growth conditions and should show whether NssR regulates these genes directly or indirectly, by modulating the expression of other regulator(s); this approach could also aid the identification of transcriptional start points associated with NssR regulation. The ChIP will be carried out in two different ways; firstly a polyclonal antibody will be raised to His•tagged NssR protein expressed and purified from *E. coli* and used to immunoprecipitate NssR cross-linked to DNA in chromatin prepared from 11168-O WT. Secondly, a FLAG•tagged version of the *nssR* gene from 11168-O WT will be inserted onto the 11168-O WT chromosome in the original location, replacing the wild-type gene. Having ensured that expression of the *nssR* gene in this strain is not disrupted and that there are no evident phenotypic effects, a commercially available monoclonal antibody to the FLAG•tag will be used to immunoprecipitate DNA cross-linked to the NssR protein from 11168-O WT chromatin. Although these two approaches increase the workload required to perform



this analysis, it should allow certain technical difficulties to be avoided, such as lack of specificity of the polyclonal antibody and, if both approaches are successful, will allow the results obtained to be verified. An electrophoretic mobility shift assay will also be used to examine the ability of NssR to bind to a fragment of DNA containing the NssR binding sequence, in the absence of exogenously applied nitrosative stress.

## 3.2 Methods

### 3.2.1 Isolation of total RNA

Total RNA for analysis by qualitative reverse- transcription PCR (RT-PCR) or quantitative Real-Time PCR (qPCR) was isolated from 11168-O WT cells using a Qiagen RNeasy Plus Mini Kit (Qiagen, Crawley, UK) according to the manufacturers' instructions. Overnight liquid cultures of the strain to be examined were diluted 1:1 with MH broth and grown microaerobically with shaking for 3h; these cultures were then used to inoculate fresh broth to an OD<sub>600nm</sub> of 0.08. Cultures were then grown to an OD<sub>600nm</sub> of 0.30-0.40 before 5 ml of culture was removed and mixed with 5 ml RNAprotect bacterial reagent (Qiagen, Crawley, UK), vortexed for 5 s and incubated at room temperature for 5 minutes to stabilise the RNA prior to isolation. After centrifugation at 6,000x g for 15 minutes at 4°C the pellet was resuspended in 200 µl TE buffer containing 1 mg.ml<sup>-1</sup> lysozyme, incubated for 10 minutes at room temperature and mixed by vortexing every 2 minutes. RLT buffer (700 µl) was added and mixed by vortexing, 500 µl absolute ethanol was then added and the sample transferred to an RNeasy Mini spin column, in 700 µl aliquots, and centrifuged at 10,000 rpm for 15 s. Buffer RW1 (350 µl) was added to the column and centrifuged at 10,000 rpm for 15 s. A 70 µl aliquot of buffer RDD (RNA-free DNaseI set; Qiagen, Crawley, UK) was mixed with 10 µl of DNaseI, added to the column and incubated at room temperature for 15 minutes. After incubation 350 µl buffer RW1 was added and the column centrifuged at 10,000 rpm for 15 s; this was repeated using the same column to attempt to ensure removal of all contaminating DNA. The RNeasy column was then transferred to a fresh collection tube, 500 µl of buffer RPE added and the column centrifuged at 10,000 rpm for 2 minutes. The column was then transferred to a fresh tube and centrifuged at 13,000 rpm for 1 minute. Finally, the RNeasy column was

transferred to a 1.5 ml Eppendorf tube, 50 µl RNase- free water added to the RNeasy membrane and centrifuged at 10,000 rpm for 1 minute. The concentration of RNA was determined on a NanoDrop 100A spectrophotometer and the purity examined by agarose electrophoresis; RNA was aliquoted as required and stored at -70°C. Contamination of the RNA with genomic DNA was tested for using PCR.

### 3.2.2 Reverse Transcription

Total RNA was converted to cDNA using random hexanucleotide primers (Promega, Madison, USA) and Superscript II Reverse Transcriptase (Invitrogen, Paisley, UK) according to the manufacturers' instructions. Total RNA (500 ng) was transcribed using 300 ng of random hexanucleotide primers and 1 µl 500µM dNTP's (Bioline, London, UK; 125µM per dNTP) in a 20 µl reaction volume. The reaction was heated to 65°C for 5 minutes then cooled on ice before 4 µl First-strand buffer (Invitrogen, Paisley, UK), 2 µl DTT (Invitrogen, Paisley, UK) and 40 units Rnasin Ribonuclease Inhibitor (Promega, Madison, USA) were added. The reaction was incubated at 25°C for 2 minutes after which Superscript II (200 units) was added to RT+ reactions and RNase-free water added to RT- reactions; the reaction was then incubated at 25°C for 10 minutes, 42°C for 50 minutes and 70°C for 15 minutes. The resulting cDNA was aliquoted and stored at -20°C prior to use. The reaction volume was increased proportionally depending upon the amount of RNA required.

### 3.2.3 Transcriptional mapping of the FMR cluster using qualitative reverse-transcription PCR

An analysis of transcription within the flagellin modification cluster of *C. jejuni* 11168-O WT was carried out using qualitative reverse-transcription PCR. Transcription was tested in three separate log-phase total RNA samples isolated from 11168-O WT and converted to cDNA using the method given above. The oligonucleotide primers used for real-time PCR were used to test for the presence of each gene of the FMR within the cDNA pool created from each RNA sample. The primer combinations used are given in Table 3.1 and primer sequences in Table 3.2. The transcript continuity of each gene in the FMR was also tested. Combinations of primers for every gene less than 4kb downstream of the gene being examined were tested; the primers used are also given in Table 3.1. BioMix Red (Bioline, London, UK) was used for all PCR reactions less than 2kb in length and Bio-X-act long (Bioline, London, UK) for all PCR reactions greater than 2kb. The following PCR protocols were used. Expected product size less than 1kb: 95°C 2 minutes, 30 cycles of 95°C 30s, 55°C 30s, 68°C 1 minute followed by a 5 minute final extension at 68°C. Expected product size 1-2kb: 95°C 2 minutes, 30 cycles of 95°C 30s, 55°C 30s, 68°C 2 minutes and a 5 minute final extension at 68°C. Expected product size greater than 2kb 95°C for 2 minutes, 30 cycles of 92°C 30s, 55°C 30s, 68°C 3 minutes with a +10s/cycle extension gradient added to the final 15 cycles, followed by a final extension of 7 minutes at 68°C. Amplicons representing the transcripts present in the sample were visualised using 1.0% agarose gels and stained with 0.5 µg.ml<sup>-1</sup> ethidium bromide. Negative controls for all reactions were carried out (data not shown) using RNA as a PCR template, while positive controls were carried using 11168-O WT genomic DNA.

**Table 3.1. Primer combinations used for transcript analysis.** The primers used are identical to those used for qPCR; the numbering system used here is the same as that of Table 3.2, where the primer sequences are given.

Gene(s) tested for transcript continuity	Primers used (sequences listed in Table 3.2)		Distance between primers (bp)
<b>Cj1317</b>	<b>1-2</b>	(1317F - 1317R)	178
Cj1317- Cj1318	<b>1-4</b>	(1317F - 1318/36R)	1923
Cj1317- Cj1319	<b>1-6</b>	(1317F - 1319R)	3323
<b>Cj1318<sup>I</sup></b>	<b>3-4</b>	(1318/36F-1318/36R)	145
Cj1318- Cj1319	<b>3-6</b>	(1318/36F - 1319R)	1545
Cj1318- Cj1320	<b>3-8</b>	(1318/36F - 1320R)	2346
Cj1318- Cj1321	<b>3-10</b>	(1318/36F - 1321R)	3853
<b>Cj1319</b>	<b>5-6</b>	(1319F - 1319R)	180
Cj1319- Cj1320	<b>5-8</b>	(1319F - 1320R)	981
Cj1319- Cj1321	<b>5-10</b>	(1319F - 1321R)	2488
Cj1319- Cj1322	<b>5-12</b>	(1319F - 1322R)	2710
<b>Cj1320</b>	<b>7-8</b>	(1320F - 1320R)	191
Cj1320- Cj1321	<b>7-10</b>	(1320F - 1321R)	1698
Cj1320- Cj1322	<b>7-12</b>	(1320F - 1322R)	1920
Cj1320- Cj1323	<b>7-14</b>	(1320F - 1323R)	2487
<b>Cj1321</b>	<b>9-10</b>	(1321F - 1321R)	193
Cj1321- Cj1322	<b>9-12</b>	(1321F - 1322R)	415
Cj1321- Cj1323	<b>9-14</b>	(1321F - 1323R)	982
Cj1321- Cj1324	<b>9-16</b>	(1321F - 1324R)	2031
<b>Cj1322</b>	<b>11-12</b>	(1322F - 1322R)	81
Cj1322- Cj1323	<b>11-14</b>	(1322F - 1323R)	648
Cj1322- Cj1324	<b>11-16</b>	(1322F - 1324R)	1697
Cj1322- Cj1325	<b>11-18</b>	(1322F - 1325R)	2079
<b>Cj1323</b>	<b>13-14</b>	(1323F - 1323R)	232
Cj1323- Cj1324	<b>13-16</b>	(1323F - 1324R)	1281
Cj1323- Cj1325	<b>13-18</b>	(1323F - 1325R)	1663
Cj1323- Cj1326	<b>13-20</b>	(1323F - 1326R)	2148
<b>Cj1324</b>	<b>15-16</b>	(1324F - 1324R)	134
Cj1324- Cj1325	<b>15-18</b>	(1324F - 1325R)	516
Cj1324- Cj1326	<b>15-20</b>	(1324F - 1326R)	1001
Cj1324- Cj1327	<b>15-22</b>	(1324F - 1327R)	1973
<b>Cj1325</b>	<b>17-18</b>	(1325F - 1325R)	89
Cj1325- Cj1326	<b>17-20</b>	(1325F - 1326R)	574
Cj1325- Cj1327	<b>17-22</b>	(1325F - 1327R)	1546
Cj1325- Cj1328	<b>17-24</b>	(1325F - 1328R)	1948
<b>Cj1326</b>	<b>19-20</b>	(1326 F - 1326R)	229
Cj1326- Cj1327	<b>19-22</b>	(1326 F - 1327R)	1201
Cj1326- Cj1328	<b>19-24</b>	(1326 F - 1328R)	1603
Cj1326- Cj1329	<b>19-26</b>	(1326 F - 1329R)	3067
<b>Cj1327</b>	<b>21-22</b>	(1327 F - 1327R)	188

Cj1327- Cj1328	<b>21-24</b>	(1327 F - 1328R)	590
Cj1327- Cj1329	<b>21-26</b>	(1327 F - 1329R)	2054
Cj1327- Cj1330	<b>21-28</b>	(1327 F - 1330R)	3063
<b>Cj1328</b>	<b>23-24</b>	(1328 F - 1328R)	198
Cj1328- Cj1329	<b>23-26</b>	(1328 F - 1329R)	1662
Cj1328- Cj1330	<b>23-28</b>	(1328 F - 1330R)	2671
Cj1328- Cj1331	<b>23-30</b>	(1328F - 1331R)	3379
<b>Cj1329</b>	<b>25-26</b>	(1329F - 1329R)	237
Cj1329- Cj1330	<b>25-28</b>	(1329F - 1330R)	1246
Cj1329- Cj1331	<b>25-30</b>	(1329F - 1331R)	1954
Cj1329- Cj1332	<b>25-32</b>	(1329F - 1332R)	2469
<b>Cj1330</b>	<b>27-28</b>	(1330F - 1330R)	117
Cj1330- Cj1331	<b>27-30</b>	(1330F - 1331R)	825
Cj1330- Cj1332	<b>27-32</b>	(1330F - 1332R)	1340
Cj1330- Cj1333	<b>27-34</b>	(1330F - 1333R)	3263
<b>Cj1331</b>	<b>29-30</b>	(1331F - 1331R)	163
Cj1331- Cj1332	<b>29-32</b>	(1331F - 1332R)	678
Cj1331- Cj1333	<b>29-34</b>	(1331F - 1333R)	2601
<b>Cj1332</b>	<b>31-32</b>	(1332F - 1332R)	181
Cj1332- Cj1333	<b>31-34</b>	(1332F - 1333R)	2104
<b>Cj1333</b>	<b>33-34</b>	(1333F - 1333R)	123
Cj1333- Cj1334	<b>33-36</b>	(1333F - 1334R)	2026
Cj1333- Cj1335	<b>33-38</b>	(1333F - 1335R)	2678
Cj1333- Cj1336	<b>33-4</b>	(1333F - 1318/36R)	3018
<b>Cj1334</b>	<b>35-36</b>	(1334F - 1334R)	196
Cj1334- Cj1335	<b>35-38</b>	(1334F - 1335R)	848
Cj1334- Cj1336	<b>35 - 4</b>	(1334F - 1318/36R)	1714
Cj1334- Cj1337	<b>35-40</b>	(1334F - 1337R)	3838
<b>Cj1335</b>	<b>37-38</b>	(1335F - 1335R)	87
Cj1335- Cj1336	<b>37-44</b>	(1335F - 1318/36R)	953
<b>Cj1336<sup>1</sup></b>	<b>3-4</b>	(1318/36F -1318/36R)	145
Cj1336- Cj1337	<b>3-40</b>	(1318/36F - 1337R)	2269
<b>Cj1337</b>	<b>39-40</b>	(1337F - 1337R)	121

<sup>1</sup>Cj1318 and Cj1336 are directly repeated genes; hence it is not possible to distinguish between expression of these genes.

### 3.2.4 Relative quantification of gene expression using SYBR Green real-time PCR (qPCR).

Specific primers for quantitative real-time PCR (qPCR) were designed using the Primer 3 software (CampyDB; <http://campy.bham.ac.uk>). Primers were checked for dimer formation and suitability for real-time PCR according to the method of Livak and Schmittgen (2001); checked primer sequences are given in Table 3.2. The delta-delta  $C_T$  ( $\Delta\Delta C_T$ ) method used here is an approximate calculation which makes a critical assumption, in that the efficiencies of amplification of target and reference genes are assumed to be approximately equal (Livak and Schmittgen, 2001). To ensure the validity of carrying out relative quantification using the  $\Delta\Delta C_T$  method, a validation experiment must be carried out, which is described as follows. Genomic DNA was extracted from 11168-O WT and serially diluted three times with water; the three dilutions (200, 20 and 2ng.µl<sup>-1</sup>) were then used as templates for qPCR for all genes. The average crossing threshold ( $C_T$ ) and  $\Delta C_T$  (the difference in crossing threshold between the target and reference genes) was determined for each DNA dilution of each gene, including the control gene *gyrA*.  $C_T$  is also sometimes described as the cycle threshold, although the meaning of these two terms is identical. The DNA gyrase gene, *gyrA*, has previously been used as a control to quantify transcript levels by qPCR (Wosten *et al.*, 2004; Elvers *et al.*, 2005). The log<sub>10</sub> of template dilution was plotted against  $\Delta C_T$ , according to the manufacturers' recommendations, if the slope is less than 0.1, then the assumption (that the target and reference genes have equal amplification efficiencies) is true and the  $\Delta\Delta C_T$  method can be used for the relative quantification of target transcripts. A gradient of close to zero was obtained for the majority of genes indicating that the  $\Delta\Delta C_T$  calculation was valid for these genes (data not shown). For those genes

where the gradient obtained was not close to zero, primers were redesigned and retested until the desired result was obtained.

cDNA was used as the template for qPCR. The positive control used was the supernatant of 11168-O WT boiled for 10 minutes and centrifuged at 10,000 rpm for 5 minutes to remove all cell debris. Per reaction 12.5  $\mu$ l 2 x Rainbow SYBR Green Master Mix (Quantace), 50nM of gene-specific primers in 2  $\mu$ l total volume, 3  $\mu$ l cDNA and 7.5  $\mu$ l HPLC quality water (Fisher Loughborough, UK) were used, giving a 25  $\mu$ l total volume. Reactions were loaded into an optical 96-well microtitre plate with optical plate seal (Applied Biosystems, Foster City, USA). An ABI prism 7000 Sequence Detector (Applied Biosystems, Foster City, USA) was programmed with the following cycle: an initial denaturation step of 10 minutes at 95°C followed by 40 cycles of 15 s at 95°C and 1 minute at 60°C. Melting curve analysis following the PCR reactions was used to monitor PCR specificity and detect any mispriming and primer dimerisation.

The qPCR data generated by the ABI Prism 7700 sequence detection software is presented as plots showing fluorescence from SYBR green binding to dsDNA against the cycle number. Following adjustment of a base line of fluorescence, an arbitrary threshold was set at the beginning of the linear part of the curve, when viewed as a log plot. This gives the cycle number ( $C_T$ ) at which the fluorescence crosses the threshold, which represents the point at which the amplification of PCR products becomes exponential, allowing quantification and comparison of the amount of cDNA in different samples. Three replicates for each gene were carried out for each of the four biological replicates and exported to Excel 2007 (Microsoft) where the mean  $C_T$  value was used for statistical analysis by the method described by Livak and Schmittgen (2001). The  $\Delta C_T$  was calculated by subtracting the mean  $C_T$  value of the target gene from the mean  $C_T$  value of the endogenous control gene (*gyrA*). Next the relative fold



changes of the mRNA expression levels of each test gene were obtained using the  $2^{-\Delta\Delta C_T}$  formula:

$$\Delta\Delta C_T = (C_{T\text{target}} - C_{T\text{gyrA}})_A - (C_{T\text{target}} - C_{T\text{gyrA}})_B$$

where A and B correspond to the different test samples, 11168-O WT and 11168-O 0466<sup>-</sup> respectively, when examining expression in the mutant, and both A and B to 11168-O WT, when examining expression in that strain only. The data were expressed as fold changes  $\pm$  SD (standard deviation) for the four separate RNA samples and combined to represent the data graphically.

**Table 3.2. Gene-specific primers for qPCR**

Number	Primer Name	Sequence (5'-3')
1	1317F	CGCAGGAAGTCTTGAAATGG
2	1317R	CCATTCATAAGGGGTTTTTGC
3	1318/36F <sup>1</sup>	TTGTGCGGATTCTTCTTATCC
4	1318/36R <sup>1</sup>	GCCTTTGGATGAACAACACC
5	1319F	GGCGCTTTAATCGCTATCC
6	1319R	GGGCTTTGAGGTTGTAAAGG
7	1320F	CATGAGCCTTGCTTTATAGGC
8	1320R	TGACTTCGCAGTTTTTCATCG
9	1321F	GACAAGGGGTTATAGTGTGTCC
10	1321R	GACAAGGGGTTATAGTGTGTCC
11	1322F	AGACTTTGGTTTAATGGGTAAGC
12	1322R	TGTTTTTCAAATTCGTATATATTGAGC
13	1323F	CATTTGCAGTAAAGGCAATCG
14	1323R	GGCCTTTTTTAAAGATTTTTTG
15	1324F	CTGATCTAGAAGGCGAATGG
16	1324R	AGTCCATAACGCACGAAACG
17	1325F	TTTCCAGATGGTCATGTAGTACG
18	1325R	CGCTGAATGTACACCATTGC
19	1326 F	CACAGGTGGAATACTTTTTGC
20	1326 R	AAATATAATGGTGTGCCGATCC
21	1327 F	GGCCTGATCATAAAGCAAGC
22	1327 R	GGCGTTTGGTAGTAAGATTTC
23	1328 F	CCTTCAAATCATCGCTACAGG
24	1328 R	AACGATCGCCTAAAATCACC
25	1329 F	ATCATGGCTGGAGGCTTAGG
26	1329 R	CTTAAAGCCCCTGCTGTACC
27	1330 F	AGGTGGTGGGGTTTTACTCG
28	1330 R	CCAAAAAGGCAAAATCATCG
29	1331F	GATCGGACTGCAAAACTTCC
30	1331 R	AACTGCGGTGATGAGATTGG
31	1332 F	TTTGTAGCAGGAGCTTGTGG
32	1332 R	TGAAGGGCGGTTTGTAAGC
33	1333 F	TCAAGCTTACGGAGACAATGG
34	1333 R	CTATCCTTGCTCCACCTTCG
35	1334 F	TTAGCTTATGGCGAGGATGG
36	1334 R	ACCTTCGGTGCAGTTGTAGG
37	1335 F	CTTCACCCCTACCCAAAAAG

<b>38</b>	1335 R	ACCTAAGATAAGTTCAAATTTACTAGAC
<b>39</b>	1337 F	TTGGGGCTAGACGATTATGG
<b>40</b>	1337 R	ATGTGTTTGCCCATCTTTGG
<b>41</b>	gyrAF	ATGCTCTTTGCAGTAACCAAAAAA
<b>42</b>	gyrAR	GGCCGATTTACGCACTTTA

<sup>1</sup> Primers for Cj1318 and Cj1336 are identical as these genes are direct repeats of one another, therefore it is impossible to distinguish between levels of expression of these genes by qPCR and any result obtained is an average expression level.

### **3.2.5 5'Rapid Amplification of cDNA Ends (RACE) to identify transcriptional start points in the FMR.**

5'RACE is a technique used for the amplification of nucleic acid sequences from an mRNA template between a defined internal sequence and the unknown sequence at the 5' end of the mRNA, allowing the identification of the transcriptional start point of the mRNA. The first step in 5'RACE is the synthesis of cDNA using a gene-specific antisense oligonucleotide primer (GSPI). The cDNA is then purified to remove primers and any unincorporated dNTPs. Terminal deoxynucleotide transferase (TdT) is used to add a homopolymeric tail to the 3' end of the cDNA, allowing amplification of the cDNA using a nested gene-specific primer (GSPII) which binds 3' to GSPI and an anchor primer which anneals to the homopolymeric tail. This amplification can be repeated if necessary using a third gene-specific primer (GSPIII), located 3' of the GSP II. The PCR product can then be sequenced directly or after cloning into a vector, allowing the transcriptional start point to be identified from sequence analysis. In this study 5'RACE was used to attempt to identify any transcriptional start points associated with potential NssR binding sites in the FMR. A 5'RACE kit (version 2.0, Invitrogen, Paisley, UK) was used together with total RNA extracted from 11168-O WT; the primers used are listed in Table 3.3.

**Table 3.3. Primers used in 5' RACE.**

Gene	Primer Name	Primer Sequence (5'-3')	Position <sup>1</sup>
1318	Cj1318RACE1 (GspI)	GCAACTCTTTAATGGGATCT	Cj1318 + 222
	Cj1318RACE2 (GspII)	GATATCTAGATTATCTTTACCTAA	Cj1318 + 137
	Cj1318RACE3 (GspIII)	ATATTGTTTAAAGCCTCAA	Cj1318 + 61
1319	Cj1319Race1 (GspI)	ACTAAATTTGAACCGATAATC	Cj1319 + 324
	Cj1319Race1 (GspII)	CCTCTGCGTACTCAATTTGAAC	Cj1319 + 168
	Cj1319Race1 (GspIII)	None - no suitable sequence available, GSPII used in place.	
<i>ptmG</i> (Cj1324)	Cj1324 (GspI)	TGAAGCATCTAAGTCTAT	<i>ptmG</i> + 355
	Cj1324 (GspII)	CGACTTGCCAATGATCGGACTGA	<i>ptmG</i> + 240
	Cj1324 (GspIII)	CGATGATGAAATCGCAAGTGTAAG C	<i>ptmG</i> + 195
<i>ptmH</i> (Cj1325/6)	Cj1325Race1 (GspI)	TGTTCTATCCCATTCGCCTT	<i>ptmH</i> + 358
	Cj1325Race1 (GspII)	GAAATACCCTAAATTTGGTCATGC	<i>ptmH</i> + 279
	Cj1325Race1 (GspIII)	GTAGAAAAACATTACAATATGG	<i>ptmH</i> + 94
<i>ptmB</i> (Cj1331)	Cj1331 (GspI)	AGCTAATGATATGACAAC	Cj1331 + 312
	Cj1331 (GspII)	CGAGTGCATATCATGAAAGAAAAA CTTGG	Cj1331 + 219
	Cj1331 (GspIII)	CGATGATTGTGCTATTGCCGTAAG TGG	Cj1331 + 162

<sup>1</sup> Position relative to the predicted translational start of gene (CampyDB)

#### 3.2.5.1 First strand cDNA synthesis

First strand cDNA synthesis was carried out by adding 1 µl gene specific primer I (GSPI; 2.5µM), 5 µg total RNA and RNase-free water to a volume of 15.5 µl to an RNase-free Eppendorf tube and incubating at 70°C for 10 minutes to denature RNA. The reaction mixture was then chilled on ice and 2.5 µl 10x PCR buffer, 2.5 µl MgCl<sub>2</sub> (25mM), 1 µl dNTP mix (10mM) and 2.5 µl DTT (0.1M) added and incubated at 42°C for 1 minute. SuperScript II reverse transcriptase (1 µl; 50 units) was added and the reaction incubated at 42°C for 1h; the reaction was then incubated at 70°C for 15 minutes to terminate cDNA synthesis. Following incubation 1µl of RNase mix was added and the reaction incubated at 37°C for 30 minutes to remove the template RNA. The first strand cDNA was purified using a QIAquick PCR Purification Kit (Qiagen, Crawley, UK) and stored at -20°C prior to TdT tailing.

#### 3.2.5.2 TdT tailing of cDNA

An oligo-dC tail was added to the cDNA using TdT. Purified cDNA (10 µl) was added to 6.5 µl DNase-free water, 5 µl 5x tailing buffer and 2.5 µl dCTP (2mM) and incubated at 94°C for 3 minutes, the reaction was then chilled on ice for 1 minute. TdT (1 µl) was added and the reaction incubated at 37 °C for 10 minutes, heat inactivated at 65 °C for 10 minutes and stored at -20 °C, if necessary, prior to PCR.

#### 3.2.5.3 PCR of dC-tailed cDNA

Tailed cDNA was amplified directly by PCR. A thermal cycler (Eppendorf, Mastercycler Gradient) was equilibrated to 94 °C and the following components added to a thin-walled PCR tube kept on ice: 31.5 µl sterile distilled water, 5 µl 10x PCR buffer, 3 µl MgCl<sub>2</sub> (25mM), 1 µl dNTP mix (10mM), 2 µl GSP II (10µM), 2 µl abridged

anchor primer (10 $\mu$ M) and 5  $\mu$ l dC-tailed cDNA. *Taq* polymerase (0.5  $\mu$ l; 5U.  $\mu$ l<sup>-1</sup>) was added immediately before mixing and the tube placed into the thermal cycler; the PCR protocol detailed below was used.

1. Heating at 94°C for 2 minutes
2. 35 cycles
  1. Denaturation at 94°C for 1 minute
  2. Annealing 55°C for 1 minute
  3. Extension at 72°C for 3 minutes
  4. Final extension at 72°C for 10 minutes

Following PCR, 10  $\mu$ l of product was analysed using agarose gel electrophoresis and the remainder of the reaction stored at -20 °C prior to nested amplification.

#### 3.2.5.4 Nested amplification

Re-amplification using a dilution of the original PCR and a nested gene specific primer was used to generate sufficient product to be visible by agarose gel electrophoresis, as a single PCR of 35 cycles was not always sufficient. A 5  $\mu$ l aliquot of the original PCR was diluted with 495  $\mu$ l of TE buffer (pH 8.0). A thermal cycler (Techne) was equilibrated to 94°C and the following components added to a thin walled PCR tube kept on ice: 33.5  $\mu$ l sterile distilled water, 5  $\mu$ l 10x PCR buffer, 3  $\mu$ l MgCl<sub>2</sub> (25mM), 1  $\mu$ l dNTP mix (10mM), 1  $\mu$ l nested GSP III (10 $\mu$ M), 2  $\mu$ l abridged anchor primer (10 $\mu$ M) and 5  $\mu$ l dilution of primary PCR product. *Taq* DNA polymerase (0.5  $\mu$ l; 5U. $\mu$ l<sup>-1</sup>) was added, the tube placed in the thermal cycler and 35 cycles of PCR carried out. Following PCR, 10  $\mu$ l of product was analysed using agarose gel electrophoresis and the product stored at -20 °C. Any suitable sharp band(s) produced were purified

from the agarose gel using a Qiagen Gel Extraction Kit (Qiagen, Crawley, UK) and sequenced. If no bands were visible or a heterologous smear was present, a further round of nested PCR was carried out, using the same primers and protocol detailed above. Positive and negative controls for the various stages were carried out according to the manufacturers' instructions (data not shown).

### **3.2.6 Sequencing of upstream regions of *nssR* (Cj0466), Cj1318 and Cj1319.**

The regions of DNA upstream of Cj0466 (*nssR*), Cj1318 and Cj1319 were amplified from both *C. jejuni* 11168-O WT and 11168-GS WT genomic DNA by PCR, using the oligonucleotide primers detailed in Table 3.4. PCR products were sequenced in both directions and compared to the relevant parts of the genome sequence obtained from CampyDB (<http://campy.bham.ac.uk>) to identify any changes in sequence, particularly in the homopolymeric tract upstream of Cj1318. PCR products were tested for and quantified by agarose gel electrophoresis and purified prior to sequencing using a QIAquick PCR Purification Kit (Qiagen, Crawley, UK).

### **3.2.7 Testing for inversion between Cj1318 and Cj1336**

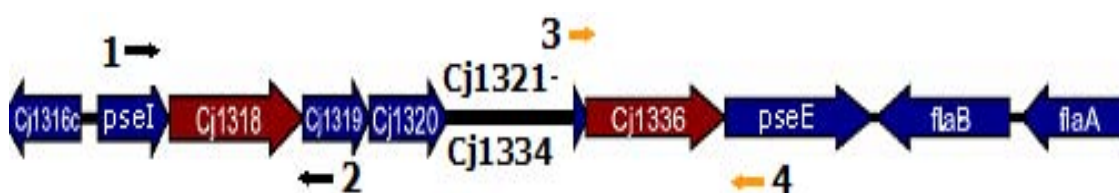
Possible inversion between the directly repeated genes Cj1318 and Cj1336 was tested for using oligonucleotide primers 1-4, detailed in Table 3.4 and Figure 3.1, in both the 11168-O WT and 11168-GS WT strains. Primers 1 and 2 are located 5' to and 3' to Cj1318 respectively, primers 3 and 4 are located 5' to and 3' to Cj1336 respectively. Primers 1 & 2 and 3 & 4 were used as pairs to test for the presence of the region in the orientation shown in the genome sequence. Primers 1 & 3 and 2 & 4 were used in pairs to test for presence of the region in the opposite orientation. Locations of the primers are shown in Figure 3.1.



**Table 3.4 Primers used in amplification, sequencing and inversion testing.**

Oligonucleotide sequence written 5' to 3'. <sup>1</sup> Position relative to predicted start codon unless stated.

Primer	Sequence	Position <sup>1</sup>
Cj1318fseq	GCGATGCTTTAAAACAAGGAG	Cj1318 -749
Cj1318rseq	TGCAACACTAAGTCCTACAGCA	Cj1318 + 838
Cj1319fseq	CCAAAAGATGCTCTTCAAGGC	Cj1319+624
Cj1319rseq	TCTCCACTTATAACTTCCATGTC	Cj1319 +172
Cj0466fseq	ATCACCTTCGCCTAAAAGCA	Cj0466 -218
Cj0466rseq	TCAAAAACAGCATTAGCAGGA	Cj0466 stop+285
Cj1318invf (1)	ATGAAAAGGTGCTTAAAAACCG	Cj1318 -205
Cj1318invr (2)	ACCTATAAAACCATCAGCACC	Cj1318 stop +38
Cj1336invf (3)	ATTGGTTTATCCAAGGTTTAGG	Cj1336 -276
Cj1336invr (4)	AAGTTGTTATACTCAAAACCGC	Cj1336 stop +61



**Figure 3.1. Relative locations of inversion primers.** Primer 1, Cj1318invF; 2, Cj1318invR; 3, Cj1336invF and 4, Cj1336invR (adapted from CampyDB; <http://campy.bham.ac.uk>).

### 3.2.8 Purification of the NssR protein.

The *nssR* gene was to be cloned from *C. jejuni* 11168-O WT, His•tagged and expressed in *E. coli*, allowing a polyclonal antibody to NssR for use in chromatin immunoprecipitation to be raised.

#### 3.2.8.1 Construction of NssR-His6 expression plasmid

The *nssR* (Cj0466) gene was amplified from *C. jejuni* 11168-O WT genomic DNA using the oligonucleotide primers given in Table 3.5.

**Table 3.5 Primers used for the cloning of *nssR*.** Bold letter indicates the base changed to remove the stop codon following *nssR*.

Name	Sequence (5'-3')	Restriction enzyme	T <sub>m</sub> (°C)
0466F- NcoI	GCGCCCATGGTAATGAAAGA TTATTTAGAAC	<i>NcoI</i>	60.0
0466R- HindIII	GCGCAAGCTTTG <b>CC</b> CAAAGT TCTTTCATGA	<i>HindIII</i>	66.9

The resulting 598bp fragment was digested with the appropriate restriction enzymes and cloned into the expression vector pET22b (+) (Novagen, Darmstadt, Germany), between the *NcoI* and *HindIII* sites and transformed into *E. coli* DH5 $\alpha$ . The gene was cloned in frame with the C-terminal 6x His•Tag, allowing protein purification by Ni<sup>2+</sup> affinity chromatography. The stop codon following the *nssR* coding sequence was mutated into a serine residue by altering the A residue in the gene sequence to a C residue in the primers, such that the C-terminal His•Tag was expressed fused with the

NssR protein. Clones were selected by screening on ampicillin plates and checked by restriction digestion and sequencing.

#### 3.2.8.2 Production of NssR-His6 fusion protein

The plasmid generated in the previous step, designated pETNssR, was transformed into the general purpose expression host *E. coli* BL21 (DE3). A starter culture of the transformed strain was prepared by inoculating 6 ml LB broth supplemented with 50  $\mu\text{g}\cdot\text{ml}^{-1}$  ampicillin with a single colony from a plate or 20  $\mu\text{l}$  of glycerol stock. This starter culture was then incubated at 37°C with shaking at 200 rpm until an OD<sub>600nm</sub> of approximately 0.5 was reached. The entire 6 ml starter culture was then added to 200 ml of LB broth, supplemented with ampicillin, and incubated as above. Once an OD<sub>600nm</sub> of 1.5 was achieved, the culture was centrifuged at 10 000 x g for 10 minutes and the pellet resuspended in an equal volume of fresh LB broth supplemented with ampicillin and IPTG to a final concentration of 1mM. This expression culture was incubated at 20°C overnight with shaking at 200 rpm. After incubation induced cells were harvested by centrifugation at 10,000 x g for 15 minutes at 4°C and stored as a pellet at -20°C.

#### 3.2.8.3 Purification of the NssR-His6 fusion protein by Ni<sup>2+</sup> Chromatography

The NssR-His6 fusion protein was purified from *E. coli* BL21 (DE3) using His•Bind 900 cartridges (Novagen, Darmstadt, Germany). The cartridges are large diameter cellulose matrix cartridges pre-charged with Ni<sup>2+</sup> which allow a higher flow rate than resin based columns. The cartridges are capable of binding 2.0 mg of His•tagged protein, which is estimated from SDS-PAGE analysis to be the amount of protein produced in approximately 100 ml cells after induction using the method above

which was not trapped in inclusion bodies. The purification method used was adapted from the manufacturers' instructions and the buffers used are detailed in Appendix 2. The harvested cell pellets were resuspended in 17.5 ml sterile distilled water, 100  $\mu$ l of 15 mg.ml<sup>-1</sup> lysozyme added and incubated at 30°C for 15 minutes. After incubation 2.5 ml 8 x binding buffer was added and the suspension sonicated at 20% amplitude (Soniprep 150 sonicator (MSE)) for 2 x 30s on ice, to break up any un-lysed cells. The lysate was centrifuged at 15,000 x g for 20 minutes to remove cell debris; at this point samples were taken from both the pellet and supernatant for analysis by SDS-PAGE. The supernatant was passed through a 0.45  $\mu$ m syringe filter (Millipore) to remove any remaining debris and prevent clogging of the column, a sample of the filtered supernatant was then collected for SDS-PAGE analysis. The column was prepared by equilibration with 6 ml 1 x binding buffer, which was pushed through the column at a rate of two drops per second using a luer-fitting syringe. The filtered cell extract was then loaded onto the column, as detailed above, washed with 4 x 5 ml, 1 x binding buffer, 2 x 5 ml wash buffer and finally eluted in 2 x 2 ml elute buffer; samples were taken after each stage for analysis by SDS-PAGE. The calculated mass of the protein with the 6 x His•Tag was 23.2 kDa.

#### 3.2.8.4 Preparation of NssR protein for polyclonal antibody production

The NssR protein was purified and concentrated prior to preparation of a polyclonal antibody to be used in ChIP-chip. The eluted protein was placed into dialysis tubing with a molecular weight cut off of 12-14 kDa. To concentrate the protein, solid PEG-15,000 was sprinkled onto the dialysis tubing and left for 1h. After this, a sample of the protein was removed for SDS-PAGE analysis and the protein dialysed into 200mM NaCl 20mM Tris-HCl (pH 7.9) overnight at 4°C. Overall, four rounds of

concentration and dialysis were required to produce protein of the required concentration for polyclonal antibody production.

### **3.2.9 Polyclonal antibody production.**

Polyclonal antibodies to the NssR protein were produced in two rabbits by Covalab ltd. (Villeurbanne, France). A total of 3 ml of NssR protein antigen, at a concentration of  $200\ \mu\text{g}.\text{ml}^{-1}$ , was provided. Per injection, 50  $\mu\text{g}$  of antigen was required in a volume of less than 500  $\mu\text{l}$ , as there were four injections per animal and two animals a total of 400  $\mu\text{g}$  of antigen was required. The immunisation protocol for both animals is given in Table 3.6. The antibodies produced were checked by Western blotting for effectiveness and cross-reactivity.

**Table 3.6. The immunisation protocol for production of polyclonal antibodies to NssR**

Day	Action
0	Pre-immune bleed (4 - 5 ml per rabbit)
0	Injection 1 (0.5 ml antigen + 0.5 ml complete Freund's adjuvant per animal)
21	Injection 2 (0.5 ml antigen + 0.5 ml incomplete Freund's adjuvant per animal)
42	Injection 3 (0.5 ml antigen + 0.5 ml incomplete Freund's adjuvant per animal)
53	Test bleed (4 - 5 ml per rabbit)
63	Injection 4 (0.5 ml antigen + 0.5 ml incomplete Freund's adjuvant per animal)
74	Test bleed (12 - 15 ml per rabbit)
88	Final bleed

### 3.2.9.1 Antibody purification

The polyclonal antibody to NssR was purified and concentrated using a 5 ml gravity flow column (Pierce, #29925) packed with 2 ml 50% UltraLink immobilised protein A/G beads (Pierce) in 1 x TBS. The column was equilibrated with 5 ml IP buffer (all buffers are detailed in section 3.2.12.1, ChIP), 10 ml antibody serum was added to 10 ml IP buffer, centrifuged for 20 minutes at 10 000 x g and the supernatant added and the column and allowed to drain through. The column was washed with 15 ml IP buffer, 15 ml IP salt buffer and eluted with 2 ml elution buffer (0.1M Glycine pH 3.0). The eluted antibody was dialysed into 0.05M Tris buffer (pH 8.0) with 0.02% sodium azide and 1% BSA and stored at 4°C.

### **3.2.10 Western Blotting**

Western blots were used to assess the purity and specificity of polyclonal antibodies raised to the NssR protein; the buffers used are detailed in Appendix 2. Proteins were separated by SDS-PAGE on a 12.5% polyacrylamide gel, including a pre-stained molecular weight marker, and transferred to a nitrocellulose membrane (Amersham Biosciences, Little Chalfont, UK) by transverse electrophoresis in 1 x Western blot buffer at 300 mA for 3 h or at 100 mA overnight, as required. The membrane was washed with 1 x TBST for 5 minutes with gentle agitation then incubated for 1h in blocking solution with gentle agitation at 4°C. The membrane was then washed in TBST for 30s and incubated in a 1:500 dilution of the primary anti-NssR antibody in blocking solution for 2h at room-temperature with gentle agitation. The membrane was then washed in 1 x TBST for 5 minutes then incubated in a 1:10,000 dilution of the secondary antibody in blocking buffer for 1 hour with gentle shaking. The membrane was washed three times in 1 x TBST for 5 minutes each time and

incubated in 5-Bromo-4-chloro-3-indolyl phosphate/ nitro blue tetrazolium (BCIP/NBT) solution prepared from Sigma Fast BCIP/NBT tablets (Sigma, Gillingham, UK) until the colour had developed to the desired level. A wash with 1% acetic acid was used to stop the reaction. The membrane was scanned using a BioRad GS-710 Densitometer.

#### Primary antibody

Polyclonal antibody raised in Rabbit against purified NssR protein (Section 3.2.9.1).

#### Secondary antibody

Anti-Rabbit IgG (whole molecule)- Alkaline phosphatase conjugate antibody produced in goat (Sigma, Gillingham, UK; product number: A3687).

### **3.2.11 Flag tagging NssR for ChIP**

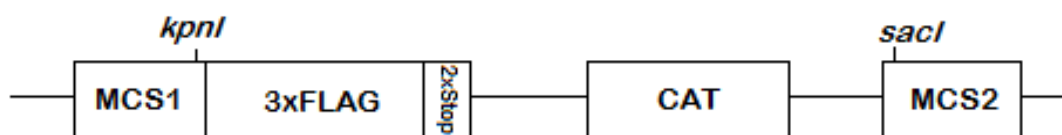
As an alternative to the previous approach which involved raising a polyclonal antibody to purified His-tagged NssR and as a method to provide confirmation of any results generated, a copy of the *nssR* gene was fused to a sequence encoding the FLAG protein tag and transferred to the 11168-O WT chromosome, to replace the wild-type gene. This allowed chromatin immunoprecipitation to be carried out using a monoclonal, commercially produced, anti-FLAG antibody, which should be more efficient and specific than a polyclonal antibody.



### 3.2.11.1 Preparation of the *nssR*-FLAG construct.

The plasmid pDEX\_Fcc was used to produce the *nssR*-FLAG fusion sequence which was transferred to the 11168-O WT chromosome in the original position of the *nssR* gene (for the sequence of pDEX\_Fcc see Appendix one). The locations of the multiple cloning sites in relation to the FLAG-tag sequence and antibiotic resistance gene are detailed in Figure 3.2.

**Figure 3.2. pDEX\_Fcc.** Location of multiple cloning sites (MCS), 3 x FLAG-tag sequence and chloramphenicol resistance gene (*cat*).



The *nssR* sequence was cloned into the *kpnI* site in MCS1, in-frame with the FLAG sequence and with the stop codon following the *nssR* gene, which was mutated to a serine residue by altering a base in the reverse primer, creating a translational fusion of the NssR protein with the C-terminal FLAG-tag. The Cj0467 sequence was cloned into the *sacI* restriction site in MCS2 to provide the second, downstream, sequence allowing homologous recombination to occur. This allows the transfer of the NssR-FLAG fusion sequence and the *cat* gene, conferring resistance to chloramphenicol, to the chromosome in the original position of the *nssR* gene, replacing the wild-type sequence.

**Table 3.7. Primers used for amplification of cloned regions**

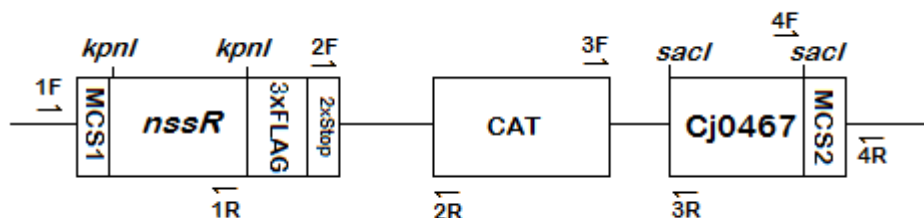
Name	Sequence (5' → 3')
nssR_kpnI_F	GCGCGGTACCATGAAAGATTATTTAGAACT
nssR_kpnI_R	GCGCGGTACCTTCCCAAAGTTCTTTCATG
Cj0467_sacI_F	GCGCGAGCTCAATTTAAGGTAGAGAATGG
Cj0467_sacI_R	GCGCGAGCTCGTTCAAGCATGAAGCAACC

**Table 3.8 Primers used to check the plasmid construct by PCR and sequencing.**

**Primer locations shown in Figure 3.3.**

Name	Sequence (5' → 3')
pDEX_Fcc_1F_vector	GGGTAATCGATGAATTCAAGC
pDEX_Fcc_2F_FLAG	CGACTACAAAGATGACGACG
pDEX_Fcc_3F_cat	GGATGAATTACAAGACTTGC
pDEX_Fcc_4F_0467	CCTATAAGAACAGATGAGCTA
pDEX_Fcc_1R_nssR	CACTCGAGATAAAGATTGCG
pDEX_Fcc_2R_cat	TACCGTCATACTATATGTGC
pDEX_Fcc_3R_0467	TTATCGTAAGCCAAGCAGCC
pDEX_Fcc_4R_vector	CGTTGTAAAACGACGGCCAG

**Figure 3.3. The final construct, pDEX\_Fcc\_nssR\_Cj0467.** Showing location of inserts and sequencing primers.



The *nssR* and Cj0467 sequences were amplified from 11168-O WT genomic DNA using BIO-X-ACT long (Bioline, London, UK) and the primers detailed in Table 3.7. PCR reactions were checked by agarose gel electrophoresis to ensure that a single, homogeneous, fragment was produced. The *nssR* fragment was digested with *kpn*I (NEB, Hitchin, UK) and the Cj0467 fragment with *sac*I (NEB, Hitchin, UK); both fragments were purified using a Qiagen PCR purification kit (Qiagen, Crawley, UK). Next, the pDEX\_Fcc plasmid was digested with *kpn*I, treated with calf intestinal alkaline phosphatase (CIP), the *nssR* fragment ligated into the plasmid and the ligation transformed into *E. coli* DH5 $\alpha$  to create the pDEX\_Fcc-*nssR* plasmid. A total of twenty two chloramphenicol resistant clones were recovered and analysed by colony PCR, using primers 1F\_vector and 2R\_cat (Table 3.8). Colonies showing the correct PCR product size (860bp) were grown in LB broth and the plasmids isolated using a Plasmid Midi kit (Qiagen, Crawley, UK). The sequence of the pDEX\_Fcc-*nssR* plasmid was confirmed by DNA sequencing using primers 1F\_vector, 2F\_FLAG, 1R\_*nssR* and 2R\_cat.

The pDEX\_Fcc-*nssR* plasmid was digested with *sac*I and treated with CIP prior to ligation of the Cj0467 fragment and transformation into *E. coli* DH5 $\alpha$  to create the pDEX\_Fcc-*nssR*-Cj0467 plasmid. Chloramphenicol resistant clones were isolated and

checked by colony PCR, using primers 4R\_vector and 3F\_cat. A correct clone was selected, the pDEX\_Fcc\_nssR\_Cj0467 plasmid isolated using a Plasmid Midi kit (Qiagen, Crawley, UK) and confirmed by sequencing using primers 3F\_cat, 3R\_0467, 4F\_0467 and 4R\_vector.

### 3.2.11.2 Homologous recombination of NssR-FLAG into 11168-O WT

Electrocompetent *C. jejuni* 11168-O WT cells were prepared, as described in Section 2.16, and electroporated with pDEX\_Fcc\_nssR\_Cj0467. Ten chloramphenicol resistant colonies were selected at random and examined by colony PCR, using primer pairs detailed below and in Table 3.8:

- 1) *nssR*\_FLAG\_F and 2R\_cat
- 2) *nssR*\_FLAG\_F and 3R\_0467
- 3) 3R\_cat and Cj0467\_sacI\_R
- 4) Cj0467\_sacI\_F and 4R\_vector

A colony producing PCR product of the correct size for all reactions, excluding reaction 4, where no product is expected if the sequence has recombined correctly, was selected and grown in MH broth. Genomic DNA was isolated and sequenced using primers 1R\_nssR and 4F\_Cj0467 to check that the DNA sequence outside of the regions used for homologous recombination was correct. Glycerol stocks of the correct clone were produced and the strain was designated 11168-O *nssR*-FLAG.

3.2.11.3 Confirmation of *nssR* activity in 11168-O *nssR*-FLAG by light microscopy

To confirm that replacement of the wild-type *nssR* gene with an *nssR*-FLAG fusion did not alter the expression or activity of the NssR protein, the 11168-O *nssR*-FLAG strain was examined by phase-contrast light microscopy to compare its motility to that of the parent strain; 11168-O WT motility was adversely affected when the *nssR* gene was deleted, creating the 11168-O 0466<sup>-</sup> strain.

3.2.11.4 Confirmation of *nssR* activity in 11168-O *nssR*-FLAG by qPCR

The expression levels of the genes of the FMR together with *nssR* itself and Cj0467, a gene whose expression is regulated by NssR, were examined by qPCR to attempt to identify whether the addition of a FLAG-tag to NssR had any affect on its function. These expression levels were compared to those seen in the parent strain. The methods used for the qPCR analysis of the 11168-O WT, detailed in section 3.2.4, were used here. Primer sequences used to analyse *nssR* (Cj0466) and Cj0467 expression in both 11168-O WT and 11168-O *nssR*-FLAG are given in Table 3.9 and Table 3.2, section 3.2.4.

**Table 3.9 Primer sequences used for real-time qPCR**

Name	Sequence (5' → 3')
0466_F_qPCR	TGCTGTTTTTGAAGAGGATGG
0466_R_qPCR	CGAGATAAAGATTGCGCTCTAAC
0467_F_qPCR	TTAAGAGCGGGATTTGAAGC
0467_R_qPCR	ATTCACCAAATCTGGCAAGG

### 3.2.12 Chromatin immunoprecipitation (ChIP).

The buffers and solutions used here are detailed in Appendix 2.

#### 3.2.12.1 Preparation of chromatin from *C. jejuni*

MH Broth (10 ml) was inoculated with 11168-O WT and grown overnight. This culture was used to inoculate 20 ml of fresh MH broth to an OD<sub>600nm</sub> of 0.05, which was then grown until an OD<sub>600nm</sub> of approximately 0.3 was reached. To prepare chromatin, 15 ml of the 11168-O WT culture was transferred to a fresh falcon tube, 405 µl of 37% formaldehyde (Sigma, Gillingham, UK) was added (to a final concentration of 1% formaldehyde) and incubated at room-temperature for 20 minutes. 3M glycine (1.5 ml) was added and the reaction incubated for 5 minutes at room-temperature, to quench any un-reacted formaldehyde. The reaction was then centrifuged at 15,000 x g to pellet the cells which were then washed three times in 1 x TBS. Finally the cells were resuspended in 0.5 ml lysis buffer, with lysozyme, transferred to a 1.5 ml Eppendorf tube and incubated at 37°C for 30 minutes with gentle shaking. After incubation, the lysis was stopped by adding 4 ml IP buffer, then 260 µl 0.1M PMSF (to a final concentration of 1mg.ml<sup>-1</sup>) and incubating at 37°C for 10 minutes. The sample was then sonicated on ice for 2 x 30s at an amplitude of 20% using a Soniprep 150 sonicator (MSE) with a microtip probe. The chromatin was then centrifuged at 13,400 x g for 15 minutes to remove cell debris and dispensed into 800 µl aliquots. It was not found to be suitable to store chromatin either at 4°C or at -20°C as this decreased the amount of chromatin isolated, so all steps were carried out concurrently.

### 3.2.12.2 Chromatin Immunoprecipitation (ChIP).

Protein A/G beads (Pierce) were washed three times in 1 x TBS prior to use and stored as a 50% suspension in 1 x TBS. Experimental antibody<sup>+</sup> reactions were prepared containing 800 µl chromatin, 20 µl 50% Protein A/G bead suspension and 100 µl NssR polyclonal antibody (final bleed) or 5 µl monoclonal FLAG antibody (Sigma, Gillingham, UK; F7425) as appropriate. Antibody<sup>-</sup> control reactions containing 800 µl chromatin and 20 µl 50% Protein A/G beads were also set up. All reactions were incubated on a rotating wheel at room-temperature for 90 minutes, and then centrifuged at 3,000 rpm for 1 minute. The beads were transferred to a spin-x-column (Costar) and washed by resuspending them in 500 µl IP buffer, incubating on a rotating wheel for 3 minutes and centrifuging at 3,000 rpm for 1 minute. The beads were then washed with a further 500 µl IP buffer, IP salt buffer, wash buffer and finally TE buffer (pH 8.0), by resuspending, incubating for 3 minutes and centrifuging. Next the columns was transferred to a fresh 1.5 ml Eppendorf tube and the protein-DNA complexes heat eluted by adding 100 µl elution buffer to the beads and incubating for 15 min at 65°C with gentle shaking. The column was centrifuged at 3,000 rpm for 1 minute and the flow through retained. The elution was repeated with another 100 µl elution buffer and the eluate combined and the samples were transferred to 0.2 ml PCR tubes.

### 3.2.12.3 De-crosslinking

A total sample, comprising 70 µl chromatin, 10 µl 20% SDS, 20 µl 1M NaHCO<sub>3</sub> and 100 µl water, was set up and 8 µl 5M NaCl added to AB<sup>+</sup>, AB<sup>-</sup> and total samples which were then incubated overnight to reverse the formaldehyde induced DNA-protein crosslink. Next, 1 µl RNaseA (Qiagen, Crawley, UK) was added and the sample incubated at 37°C for 30 minutes. To all samples 4 µl 0.5M EDTA, 8 µl 1M Tris HCl

(pH 7.5) and 10  $\mu$ l Pronase (40mg.ml<sup>-1</sup>; Roche) were added and the samples incubated at 42°C for 2h. Finally, samples were cleaned using a QIAquick PCR Purification Kit (Qiagen, Crawley, UK), checking first to ensure that all samples were at pH  $\leq$  7.5 and 10  $\mu$ l 3M sodium acetate (pH 5) added where necessary to decrease the pH. The PCR Purification Kit protocol was carried out according to the manufacturers' instructions and DNA was resuspended in 50  $\mu$ l elution buffer. De-crosslinked total sample (5  $\mu$ l) was analysed by agarose gel electrophoresis, to check that the sizes of the sonicated DNA fragments were between 500 and 1000 bp, as required by the micro-array.

#### 3.2.12.4 Direct labelling of immunoprecipitated DNA

DNA isolated by chromatin immunoprecipitation was fluorescently labelled with either Cy3 or Cy5 Cy-dyes using the BioPrime Array CGH Genomic Labelling System (Invitrogen, Paisley, UK) and Cy3-dCTP (#PA53021) and Cy5-dCTP (#PA55021; Amersham Biosciences, Little Chalfont, UK). DNA isolated from 11168-O WT and 11168-O *nssR*-FLAG was labelled using the same method and 'reference' samples, containing total chromatin, were labelled with Cy3 and 'test' samples which had been immunoprecipitated using an antibody, were labelled with Cy5. To label 400 ng DNA in a total volume of 21  $\mu$ l, 20 $\mu$ l 2.5x random primers were added and the samples incubated at 95°C for 5 minutes. Next, 5  $\mu$ l 10x dCTP mix, 1  $\mu$ l Exo-Klenow fragment and 3  $\mu$ l of the appropriate CyDye-dCTP nucleotide was added and the samples incubated at 37°C for 3 hours; finally 5  $\mu$ l stop buffer was added. Labelled probes were purified using a QIAquick PCR Purification Kit (Qiagen, Crawley, UK) and the extent of labelling was determined using the Microarray function on a NanoDrop 1000A Spectrophotometer. Control reactions using 11168-O WT genomic DNA were carried out as necessary.



### 3.2.12.5 PCR checking of ChIP isolated DNA.

The DNA isolated by chromatin immunoprecipitation using both methods was examined by PCR. GoTaq (Promega, Madison, USA) was used, with 2.0 µl of the DNA isolated by ChIP (AB<sup>+</sup>, AB<sup>-</sup> or total sample DNA) and the products analysed by agarose gel electrophoresis. Positive controls were carried out for all primer pairs using 0.5µl of 11168-O WT DNA and negative controls using sterile distilled water. The primers used are detailed in Table 3.2 (section 3.2.4), Table 3.9 (section 3.2.11.4) and below:

*fla*\_L 5'-TTCAGTTGGAACAGGACTTGG -3'

*fla*\_R 5'-AAGGCTCCATTAGCATCACC -3'

*cgb*\_L 5'- GCTTTAGCAATGGCGATTTT -3'

*cgb*\_R 5'- CCATAAGCAACTTCCCAAGC -3'

### 3.2.13 Electrophoretic mobility shift assay of NssR and *cgb* promoter fragment

#### DNA.

An electrophoretic mobility shift assay was carried out using purified, His•tagged NssR protein and P<sup>32</sup> –labelled DNA fragments amplified from the promoter region of the *cgb* gene, which contains the consensus NssR binding site under nitrosative stress conditions, TTAAC-N<sub>4</sub>-GTAA.

#### 3.2.13.1 Preparation of the *cgb* promoter fragment

The *cgb* promoter fragment from 133bp 5' of the transcriptional start point of the gene to 64bp 3' of the transcriptional start point was amplified by PCR from 11168-O WT DNA using the following primers:

*cgb\_apal\_F* 5'-GCGCGGGCCCGCCATAGTCCCTCTTTTAA-3'

*cgb\_apal\_R* 5'-GCGCGGGCCCGGCACACAATCTTTG-3'

GoTaq DNA polymerase (Promega, Madison, USA) in a reaction volume of 200 µl was used to generate sufficient DNA for labelling. Following PCR, the product was checked by agarose gel electrophoresis to ensure that a single, homogeneous band was produced.

The PCR reaction was cleaned using a Qiagen PCR clean-up kit (Qiagen, Crawley, UK), digested using the *apaI* restriction enzyme (NEB, Hitchin, UK) and treated with calf intestinal alkaline phosphatase (NEB, Hitchin, UK) to remove the 5' phosphates, leaving the ends of the DNA free to be labelled. The fragment was purified on a 0.7% agarose gel, excised and extracted using a Qiagen gel extraction kit (Qiagen, Crawley, UK).

### 3.2.13.2 End-labelling DNA fragments with [ $\gamma^{32}$ -P] ATP.

Taking appropriate precautions for the use of radioactive compounds, a labelling mix containing 16  $\mu$ l of the *cgb* DNA fragment prepared in the previous step, 2  $\mu$ l 10x kinase buffer (NEB, Hitchin, UK), 1  $\mu$ l [ $\gamma^{32}$ -P] ATP (Amersham Biosciences, Little Chalfont, UK) and 1  $\mu$ l T<sub>4</sub> polynucleotide kinase, was prepared and incubated at 37°C for 30 minutes.

### 3.2.13.3 Removal of unincorporated nucleotides.

Unincorporated radio-nucleotides were removed by passing the labelling reaction through a Sephadex G-50 column. A 50% suspension of Sephadex G-50 in TE buffer was prepared by autoclaving 5g of dried Sephadex G-50 in 150 ml TE buffer and washing the resulting suspension three times in 150 ml sterile TE buffer; finally the Sephadex was resuspended in 50 ml TE buffer giving a 50% stock.

Two 2 ml Spin-X columns (Costar) were prepared and 400  $\mu$ l 50% Sephadex G-50 suspension added to each, the columns were then centrifuged at 3,400 rpm for 3 minutes to pack the Sephadex. The entire labelling reaction was then added to the first column and centrifuged at 3,400 rpm for 3 minutes; the flow-through was applied to the second column and centrifuged. The flow-through, containing the P<sup>32</sup>-end-labelled *cgb* DNA fragments, was aliquoted and stored at -20°C until required. The Sephadex columns, containing the unincorporated radio-nucleotides, were placed into lead pig and allowed to decay for several half lives prior to disposal.

3.2.13.4 Electrophoretic mobility shift assay

Purified NssR protein was added to the radio-labelled *cgb* DNA fragments, in a buffer designed to allow binding, then applied to an acrylamide gel to observe any retardation in the movement of the radio-labelled DNA through the gel which could be caused by the NssR protein binding the DNA. Purified NssR protein, with a final concentration of 3.2 $\mu$ M was used in the assay. Binding reactions containing a range of concentrations of NssR were used to assess the ability of NssR to bind DNA. The buffers and solutions are detailed in Appendix II.

The binding reactions contained:

$\gamma^{32}$ P-labelled <i>cgb</i> fragment (approximate concentration 50nM)	20 $\mu$ l
10x binding buffer	1 $\mu$ l
50% glycerol	1 $\mu$ l
Bovine serum albumin (10mg.ml <sup>-1</sup> )	0.5 $\mu$ l
Herring sperm DNA (500 $\mu$ g.ml <sup>-1</sup> ; Promega, Madison, USA)	1.0 $\mu$ l
Sterile distilled water	5.3 $\mu$ l

Together with 1  $\mu$ l of NssR at one of the following concentrations: 0.32 $\mu$ M, 0.16 $\mu$ M, 0.08 $\mu$ M, 0.04 $\mu$ M, 0.02 $\mu$ M or 1  $\mu$ l of sterile distilled water as a control.

As necessary reactions also contained varying concentrations of the unlabelled oligonucleotide



which contains the NssR binding site which is underlined. The concentrations used were 1 $\mu$ M, 2.5 $\mu$ M, 5 $\mu$ M, 10 $\mu$ M, 100 $\mu$ M, for these experiments the concentration of NssR remained at 0.32 $\mu$ M.

These reactions were then incubated for 10 minutes at 37°C prior to loading onto a continuous 6% acrylamide gel. The gel was pre-run for 5 minutes at 166V (12V.cm<sup>-1</sup>) in 0.25 x TBE buffer, 10µl 2x loading buffer was added to one of the wells and allowed to run into the gel for a further 5 minutes. Before loading the samples the current was turned off and the wells washed thoroughly with 0.25 x TBE buffer; the current was then reapplied and all samples loaded quickly. The gel was then run for 2h at 166V. After electrophoresis the gel was vacuum dried for 1h, placed into a phosphor-imaging cassette (Kodak) and exposed for 2h; to obtain the image the phosphor-screen was scanned using a Molecular Imager FX (BioRad, Hemel Hempstead, UK).

### 3.3 Results

#### 3.3.1 Transcript mapping of the flagella modification region by qualitative RT-PCR.

Transcript analysis by conventional qualitative reverse-transcription PCR was carried out for the FMR (Cj1318-Cj1336) and the neighbouring genes *pseI* (Cj1317) and *pseE* (Cj1337) to attempt to locate transcripts within this region in the 11168-O WT and *nssR* mutant. Analysis was carried out on three preparations of RNA from separate cultures and results were consistent for all samples. Table 3.10 details the transcripts identified in this region and Figure 3.4 shows a map of transcription within the FMR produced from this data, Figure 3.5 shows agarose gels representative of the results generated. Cj1319 and Cj1320 were apparently transcribed together, as were *ptmH* and *ptmC*, *ptmD* and *ptmE*, and *ptmB*, *ptmA* and *pseD*. *pseI* and *pseE* are outside of the FMR and appeared to be transcribed separately to it. Within the FMR Cj1318/Cj1336, Cj1321, *ptmG*, Cj1334 and were transcribed alone; no evidence for the transcription of Cj1322, Cj1323 or *ptmF* was seen using this method.

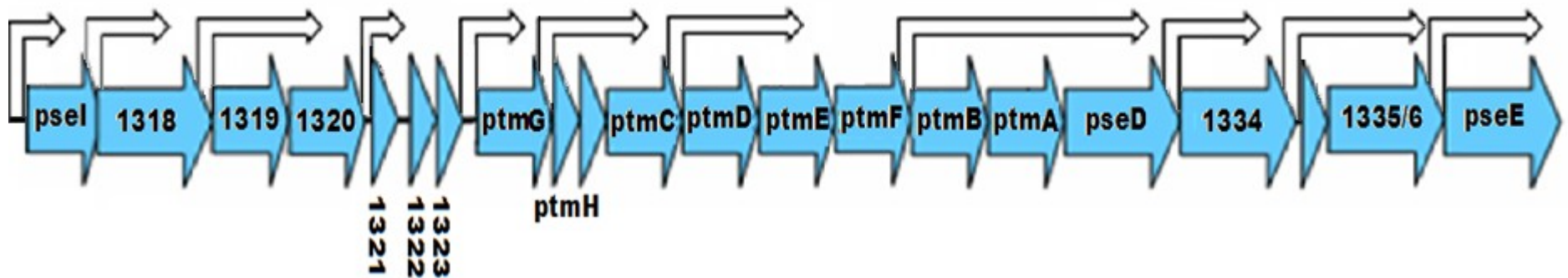
**Figure 3.10 Transcription of the FMR of *C. jejuni* 11168-O assessed by qualitative RT-PCR.** ‘+’ indicates the presence of a transcript, ‘-’ indicates the absence of a transcript between the genes tested.

Gene(s) tested for transcript continuity	Presence of transcript
<b>Cj1317</b>	+
Cj1317- Cj1318	-
Cj1317- Cj1319	-
<b>Cj1318<sup>I</sup></b>	+
Cj1318- Cj1319	-
Cj1318- Cj1320	-
Cj1318- Cj1321	-
<b>Cj1319</b>	+
Cj1319- Cj1320	+
Cj1319- Cj1321	-
Cj1319- Cj1322	-
<b>Cj1320</b>	+
Cj1320- Cj1321	-
Cj1320- Cj1322	-
Cj1320- Cj1323	-
<b>Cj1321</b>	+
Cj1321- Cj1322	-
Cj1321- Cj1323	-
Cj1321- Cj1324	-
<b>Cj1322</b>	-
Cj1322- Cj1323	-
Cj1322- Cj1324	-
Cj1322- Cj1325	-
<b>Cj1323</b>	-
Cj1323- Cj1324	-
Cj1323- Cj1325	-
Cj1323- Cj1326	-
<b>Cj1324</b>	+
Cj1324- Cj1325	-
Cj1324- Cj1326	-
Cj1324- Cj1327	-
<b>Cj1325</b>	+
Cj1325- Cj1326	+
Cj1325- Cj1327	+
Cj1325- Cj1328	-
<b>Cj1326</b>	+
Cj1326- Cj1327	+
Cj1326- Cj1328	-
Cj1326- Cj1329	-
<b>Cj1327</b>	+

Cj1327- Cj1328	-
Cj1327- Cj1329	-
Cj1327- Cj1330	-
<b>Cj1328</b>	+
Cj1328- Cj1329	+
Cj1328- Cj1330	-
Cj1328- Cj1331	-
<b>Cj1329</b>	+
Cj1329- Cj1330	-
Cj1329- Cj1331	-
Cj1329- Cj1332	-
<b>Cj1330</b>	-
Cj1330- Cj1331	-
Cj1330- Cj1332	-
Cj1330- Cj1333	-
<b>Cj1331</b>	+
Cj1331- Cj1332	+
Cj1331- Cj1333	+
<b>Cj1332</b>	+
Cj1332- Cj1333	+
<b>Cj1333</b>	+
Cj1333- Cj1334	-
Cj1333- Cj1335	-
Cj1333- Cj1336	-
<b>Cj1334</b>	+
Cj1334- Cj1335	-
Cj1334- Cj1336	-
Cj1334- Cj1337	-
<b>Cj1335</b>	+
Cj1335- Cj1336	+
<b>Cj1336<sup>1</sup></b>	+
Cj1336- Cj1337	-
<b>Cj1337</b>	+

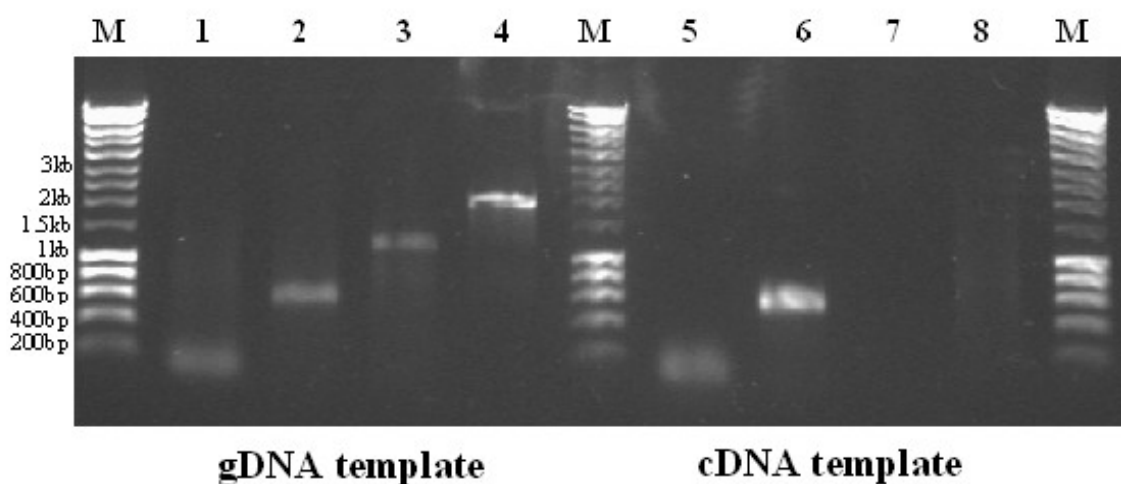
<sup>1</sup>Cj1318 and Cj1336 are directly repeated genes; hence it is not possible to distinguish between these genes.





**Figure 3.4 Transcript map of the flagella modification region and surrounding genes.** Arrows indicate the presence and extent of mRNA transcripts identified, for those genes lacking arrows, no evidence of transcription was found. Data is collated from all three repeats.

Transcription of Cj1318 and Cj1335/6 could not be individually assessed as these genes are direct repeats and therefore identical in sequence, hence it is possible that only one of these genes is in fact expressed, rather than both, as is shown above.



**Figure 3.5 Qualitative RT-PCR analysis of Cj1325 transcription.** 1.0% agarose gel stained with ethidium bromide and showing amplicons generated using gDNA (Lanes 1-4) and cDNA (Lanes 5-8) as template. Different primer combinations were used to test for the expression of multiple genes on the same transcript. Lanes 1 & 5: primers 5 & 6 used to test for transcription of Cj1319; Lanes 2 & 6: primers 5 & 8 used to test for transcription from Cj1319 to Cj1320. Lanes 3 & 7: primers 5 & 10 used to test for transcription from Cj1319 to Cj1321. Lanes 4 & 8: primers 5 & 12 used to test for transcription from Cj1319 to Cj1322. M: molecular weight markers, sizes given.

### 3.3.2 Transcriptional analysis of the flagella modification region in the 11168-O 0466<sup>-</sup> mutant by quantitative real-time PCR.

Previous work carried out by Elvers *et al.* (2005) had indicated that there were differences in gene expression in the flagella modification region between the 11168-O WT and *nssR* mutant, 11168-O 0466<sup>-</sup>, that were unrelated to exposure to nitrosative stress. Quantitative real-time PCR was carried out to examine the extent of these differences in gene expression. The DNA gyrase gene *gyrA* was used as an internal control (Wosten *et al.*, 2004; Elvers *et al.*, 2005). Growth experiments confirmed that although 11168-O WT has a slower rate of growth than 11168-O 0466<sup>-</sup> (data shown in appendix III), the cells from which RNA was extracted were in the same growth phase, hence differences observed in the real-time PCR results would be unlikely to be due to growth phase-related effects.

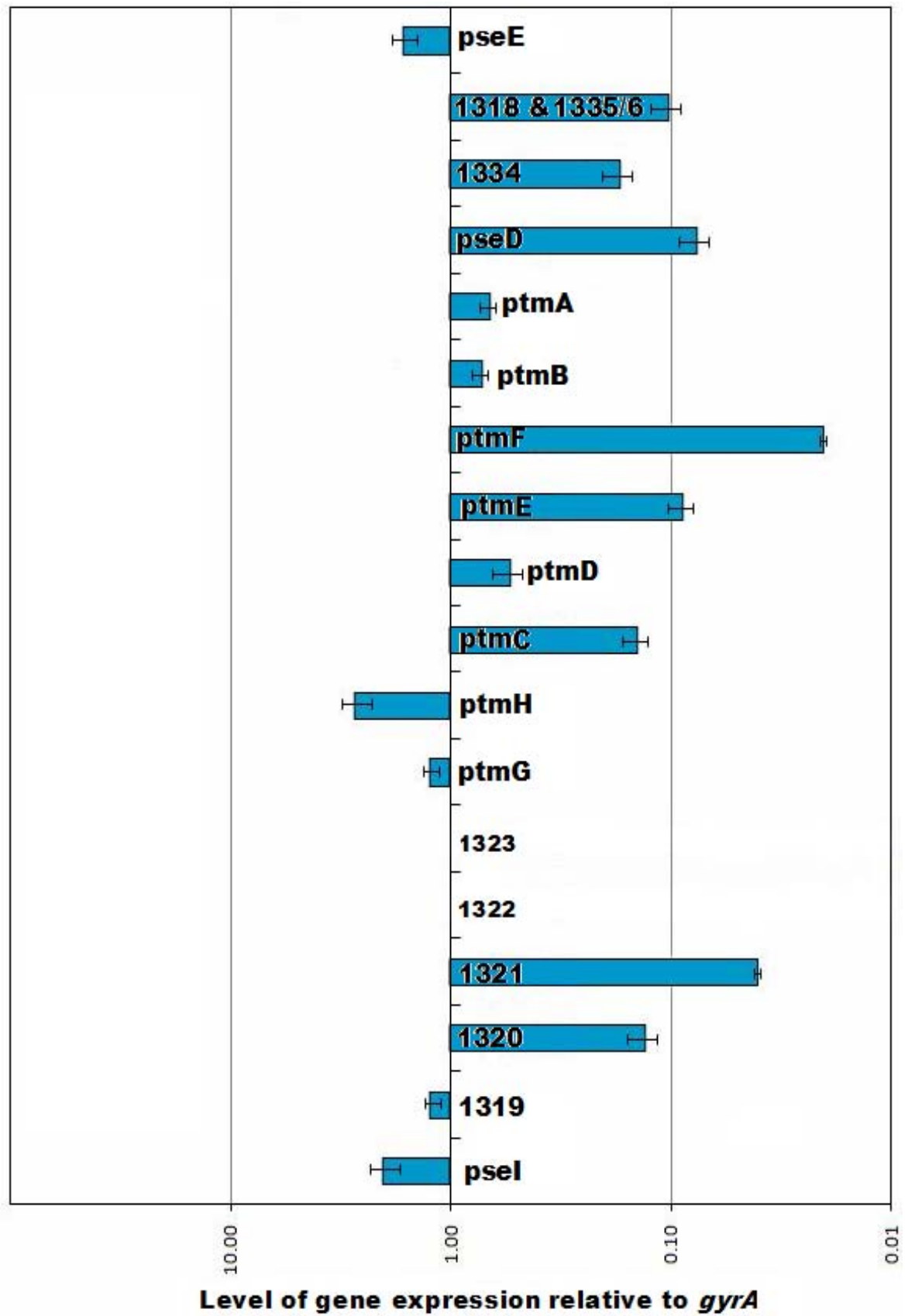
These experiments compared expression of the flagella modification region and outlying genes *pseI* (Cj1317) - *pseE* (Cj1337) in 11168-O WT to expression in 11168-O 0466<sup>-</sup>. The expression level of the genes between Cj1318 and Cj1336 in the 11168-O 0466<sup>-</sup> strain could not be quantified as the signals from all of these genes were below the limits of detection of the instrument used; this was true for each of the three biological replicates. *pseI* and *pseE* were expressed in 11168-O 0466<sup>-</sup> at levels comparable with those of the wild-type, indicating that only the flagella modification region itself (Cj1318-Cj1336) is subject to down regulation in the 11168-O 0466<sup>-</sup> strain. The selective down regulation of the FMR indicated that genes at the beginning of the region, such as Cj1318 and Cj1319, were very likely to be transcribed independently of genes just upstream of the region, such as *pseI* (Cj1317) and therefore may contain promoter sequences.

### 3.3.3 Transcriptional analysis of the flagella modification region in the 11168-O WT by quantitative real-time PCR.

These qPCR experiments examined the expression of the FMR itself within 11168-O WT by comparing gene expression in the FMR, and outlying genes *pseI* and *pseE*, in 11168-O WT to the expression of the control gene *gyrA*, allowing the level of gene expression in this region to be quantified relative to *gyrA* using the  $2^{-\Delta\Delta CT}$  method. A signal was detected for all genes except Cj1322 and Cj1323; it is possible that either these genes are not transcribed and so do not produce any signal, or that that signal was below the limits of detection of the instrument used, such the expression level of these genes could not be quantified. The levels of expression detected from the other FMR genes and calculated relative to *gyrA* are shown in Figure 3.6. Cj1318 and Cj1335/6 expression is shown together as these genes are directly repeated and hence are 100% identical in sequence, therefore it was impossible to design separate primers and only a mean level of expression could be obtained.

The difference in expression levels across the region indicates this region is unlikely to be transcribed from a single promoter. There are several hypothetical locations for promoters in this region, the major criterion being that they are located at the beginning of a region of increased expression relative to the upstream genes. Possible promoter locations could include *ptmG* (Cj1324), *ptmH* (Cj1325/6), *ptmD* (Cj1328), *ptmB* (Cj1331) and Cj1334 as these genes are located at the beginning of regions of increased expression, relative to the genes just upstream of each. There is also a possibility that the differences in mRNA levels seen here may not reflect the actual promoter activity in this region and may in fact be due to mRNA stability and degradation. Possible mechanisms could include post-transcriptional cleavage of

mRNAs seen in rRNA transcription or protection against degradation which can sometimes be conferred by the secondary structure of the mRNA.



**Figure 3.6 Quantitative real-time PCR assays of FMR gene expression in 11168-O WT.** The relative level of gene expression compared with the reference gene (*gyrA*) is shown, calculated using the  $2^{-\Delta\Delta C_T}$  method; where no bar is present no expression of the gene was observed. The error bars represent the range determined using the  $2^{-\Delta\Delta C_T}$  calculation, with  $\Delta\Delta C_{T+S}$  and  $\Delta\Delta C_{T-S}$  where S= the standard deviation of the value for  $\Delta\Delta C_T$ . These results represent one data set; the results were reproducible using separate RNA extracts from four independent cultures.

As the qPCR results demonstrated the possibility of there being transcriptional start points located upstream of Cj1318, Cj1319, *ptmG* (Cj1324), *ptmH* (Cj1325/6), *ptmD* (Cj1328), *ptmB* (Cj1331) and Cj1334, 5'RACE was used to attempt to map the 5' ends of any mRNA transcripts starting from these genes. *ptmD* and Cj1334 were excluded from this analysis as the qPCR results indicated that these genes were expressed at a lower level than the other candidates and 5'RACE has a greater chance of being successful when used to examine genes with a relatively high level of expression. Unfortunately, despite multiple attempts, no PCR products could be produced from the dt-tailed mRNA for any of the genes tested.

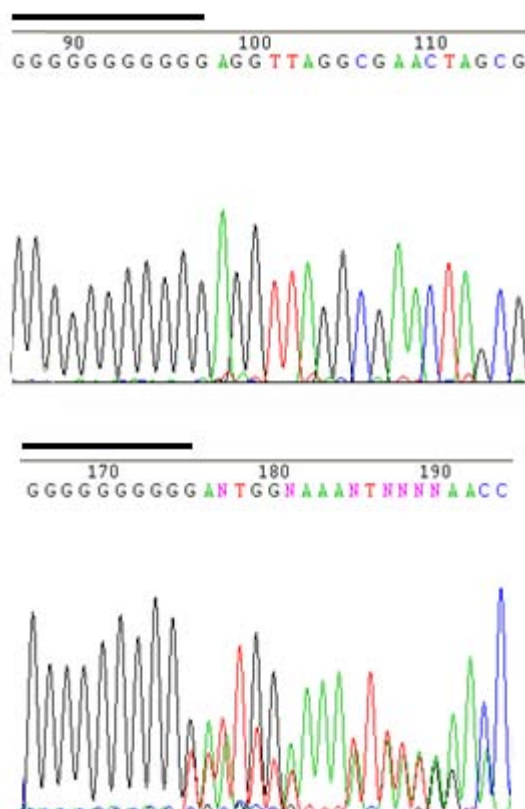
A search for the nitrosative stress dependant NssR binding sequence (consensus TTAAC-N<sub>4</sub>-GTAA) was also carried out using the Pattern Search function of CampyDB (<http://xbase.bham.ac.uk/campydb/>, accessed July 2007) . No perfect matches were found outside of the regulon regulated by NssR in response to nitrosative stress. No sites with a single mismatch were located in the FMR; however, multiple sites with two and three mismatches were located throughout the FMR, even when the search was restricted to only those potential binding sites located less than 200bp upstream of a predicted translation start point there were still too many potential sites for any to be used to aid in the identification of possible promoter regions.

### **3.3.4 Sequencing of the upstream regions of Cj0466, Cj1318 and Cj1319.**

Verification of the DNA sequences of the upstream and 5' regions of Cj0466 (*nssR*), Cj1318 and Cj1319 was undertaken in strains 11168-O WT and 11168-GS WT. The aim of this work was to discover any DNA sequence differences in areas of DNA likely to contain promoter sequences linked to these genes and so begin to elucidate the causes of the observed differences in flagellin glycosylation between these strains.



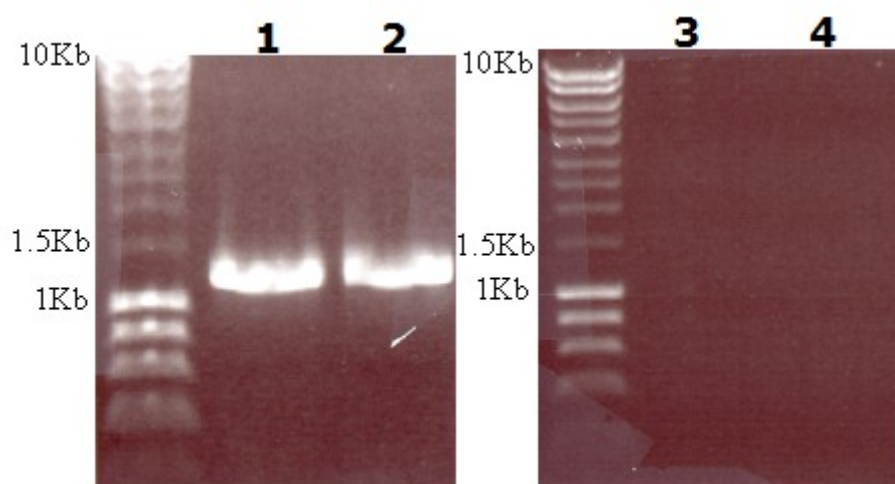
The 5' end of Cj1318 contains a homopolymeric tract (consensus = G11) located at the putative start codon +168bp. The sequence of this region was checked in both strains by PCR amplification and DNA sequencing; six biological repeats were carried out for both wild-type strains. Every sequence generated from the 11168-GS WT contained the consensus number of residues, as did five of the six sequences generated from the 11168-O WT. The DNA sequence following these sequences was 'clean' and easily legible, suggesting that the DNA template and hence the bacterial population from which these sequences were generated was homogeneous; an example of a 'clean' chromatograph is given in Figure 3.7. The sixth 11168-O WT sequence contained only ten G-residues and the following sequence was 'noisy', suggesting that the template DNA was displaced by the varying number of bases in the homopolymeric tract. As, in a non-clonal population of bacteria, there could be heterogeneity in the number of bases in a homopolymeric tract due to slipped-strand mispairing, so the DNA downstream of a tract where variation was present would appear to be staggered by one or more bases when sequenced, giving 'noisy' indistinguishable sequence which reflects the variation in the bacterial population; an example of a 'noisy' chromatograph is given in Figure 3.7. This suggests that these homopolymeric tracts are capable of variation, at least in Cj1318 in the 11168-O WT. The DNA sequences of the upstream regions of Cj1319 and *nssR* from both wild-type strains agreed in all respects with each other and the published genome sequence.



**Figure 3.7. ‘Clean’ and ‘noisy’ chromatograms generated from the sequencing of the homopolymeric tract of Cj1318.** The upper chromatogram demonstrates the ‘clean’ sequence generated from a DNA template where the homopolymeric tract does not vary and contains 11 residues. The lower chromatogram demonstrates the type of ‘noisy’ sequence generated from a population in which the homopolymeric tract contains a mixture of ten and eleven residues, with about half the population in each category, evidenced by the mixed G and T signals seen at the tenth position.

### 3.3.5 Testing for an inversion between Cj1318 and Cj1336

To test whether an inversion between the directly repeated genes Cj1318 and Cj1336 had taken place in either 11168-GS WT or 11168-O WT and could explain any of the differences in flagellin glycosylation seen between these strains, primer pairs Cj1318inv and Cj1336inv, located in unique sequences outside of the two genes, were used. When the presence of the FMR in the orientation given in the genome sequence was tested for, a band of the expected size of 1.2kb was produced, indicating that no inversion had taken place in at least in part of the population. When an inversion between Cj1318 and Cj1336 was tested for, no amplicon was produced, suggesting that inversion between these genes had not taken place in any of the bacterial population examined. Figure 3.8 shows the results of inversion testing in strains 11168-O WT and 11168-GS WT using primer pairs 1 & 2 and 1 & 3; the data for primer pairs 3 & 4 and 2 & 4 corroborates this but is not shown.



**Figure 3.8. No inversion between the direct repeats Cj1318 and Cj1336 occurs in wild-types of both backgrounds.** 0.7% agarose gels stained with ethidium bromide and showing products of inversion testing. Lane 1: product of primers 1 & 2 using template DNA from 11168-O WT. Lane 2: product of primers 1 & 2 using template DNA from 11168-GS WT. Lane 3: product of primers 1 & 3 using template DNA from 11168-O WT. Lane 4: product of primers 1 & 3 using template DNA from 11168-GS WT.

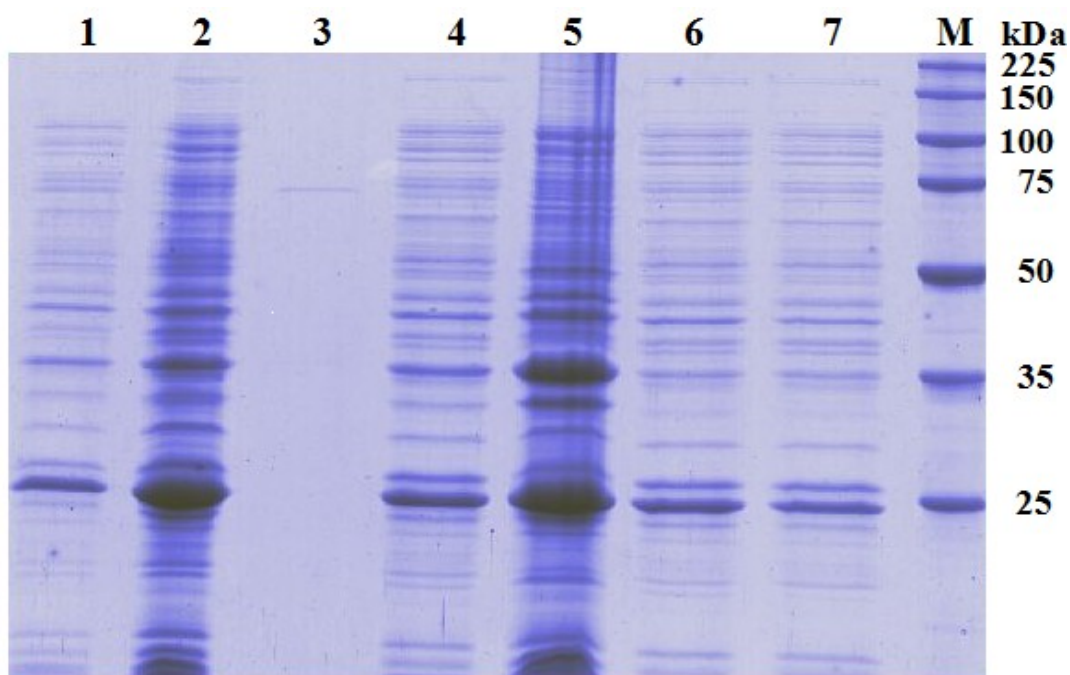
### **3.3.6. Analysis of genome-wide NssR binding by chromatin immunoprecipitation (ChIP).**

#### 3.3.6.1 Purification of the NssR protein

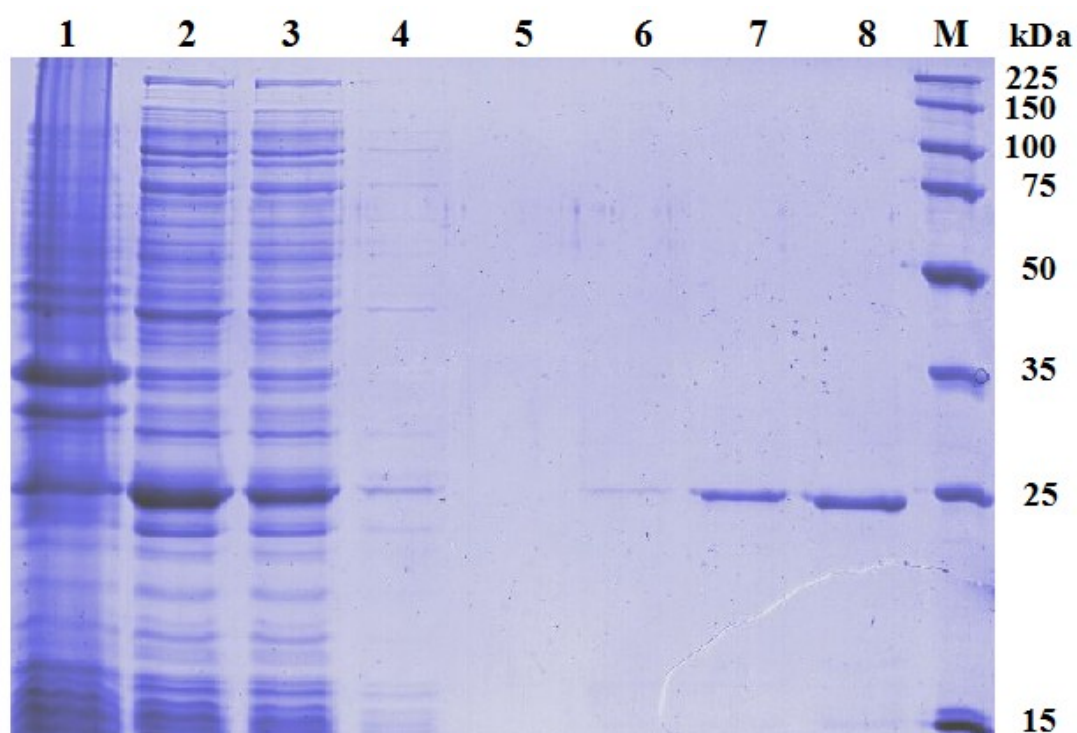
The His6-NssR protein (23.2kDa) is visible in the total protein extract from induced cells (Figure 3.9, lane 2). Insufficient protein was present in the culture supernatant to allow it to be purified so protein was obtained directly from the *E. coli* cells. Lane 5 shows that even after lysozyme treatment and sonication to lyse the cells some protein remains in the cell debris, probably in the form of inclusion bodies. Enough protein is present in the supernatant of lysed cells (lane 4) to allow purification from this fraction, rather than attempting inclusion body purification and protein re-folding. The protein was not removed by filtration with a 0.45µm syringe filter which was used to remove debris to avoid clogging of the purification cartridge. NssR was purified using Novagen His-Bind 900 cartridges (Novagen, Darmstadt, Germany) and when visualised using a Coomassie stained SDS-PAGE gel (Figure 3.10 lane 8) appeared to be homogeneous. Protein concentration was measured using the Nanodrop spectrophotometer. Following four rounds of concentration and dialysis in Tris-NaCl buffer the protein reached a concentration of 200 µg.ml<sup>-1</sup> and was at a suitable concentration for polyclonal antibody production.

#### 3.3.6.2 Preparation of a polyclonal antibody to NssR

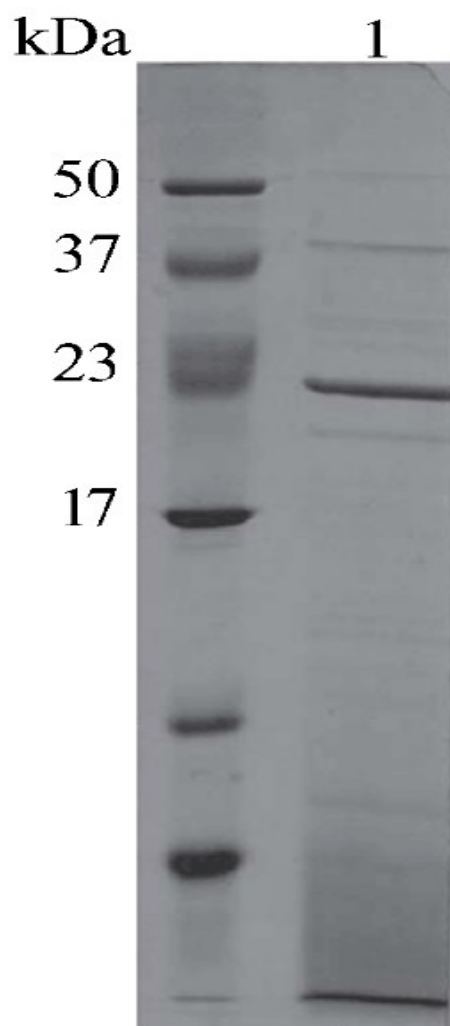
Purified, concentrated NssR protein produced in *E. coli* BL21 was used to produce polyclonal antibodies in rabbits. The antibody was then successfully purified and concentrated from the serum and its specificity tested by western blotting (Figure 3.11).



**Figure 3.9. Extraction of NssR from *E. coli* BL21(DE3) pETNssR.** Lane 1 - Culture prior to induction with IPTG. Lane 2 - Culture after induction with 1mM IPTG and incubation O/N at 20°C. Lane 3 - Culture supernatant post centrifugation. Lane 4 - Supernatant after lysozyme treatment, sonication and centrifugation to remove cell debris. Lane 5 - Cell pellet after lysozyme treatment, sonication and centrifugation to remove cell debris. Lane 6 - Supernatant prior to 0.45µm filtration. Lane 7 - Supernatant post 0.45µm filtration.



**Figure 3.10. Purification of NssR** Lane 1 - Flow through from cartridge after addition of supernatant. Lane 2 - Binding buffer wash 1. Lane 3 - Binding buffer wash 2. Lane 4 - Binding buffer washes 3 & 4. Lane 5 - Wash buffer wash 1. Lane 6 - Wash buffer wash 2. Lane 7 - Elute buffer wash 1. Lane 8 - Elute buffer wash 2.

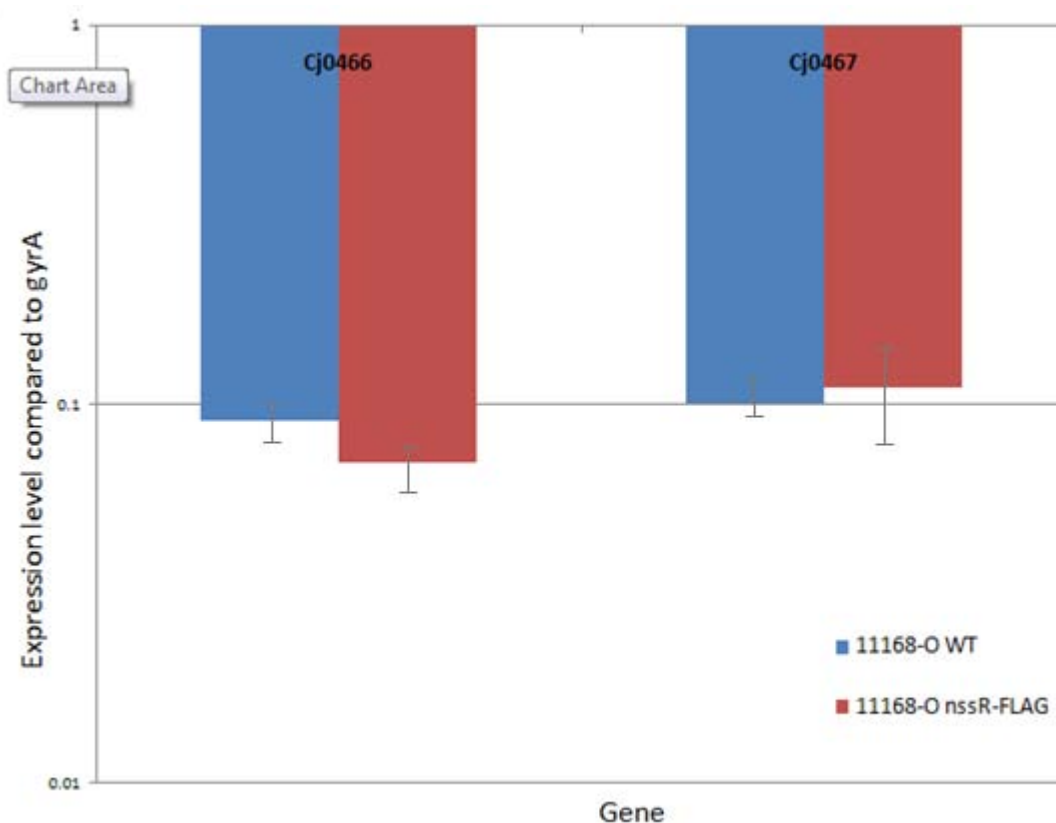


**Figure 3.11. Specificity of the polyclonal NssR antibody examined by western blotting.** Lane 1: 5  $\mu$ l boiled cell lysate from a broth culture of 11168-O WT with an  $OD_{600nm}$  of 0.2. The western blot was performed using the purified, concentrated NssR antibody as the primary antibody and demonstrates that the antibody has a high specificity for the 23.2kDa his-tagged NssR protein. The sizes of the marker proteins are shown.



### 3.3.6.3 Confirming gene expression in 11168-O *nssR*-FLAG

The sequence of the *nssR* region of the 11168-O *nssR*-FLAG strain was successfully confirmed by DNA sequencing (data not shown). The motility of the strain was examined by light microscopy and found to be indistinguishable from that of the parent strain 11168-O WT. As the *nssR* mutant strain 11168-O 0466<sup>-</sup> has a significant motility defect it can be concluded that *nssR* is being expressed in the FLAG-tagged strain and that the FLAG-tag is not impairing protein function sufficiently to cause phenotypic effects. Real-time quantitative PCR was used to compare the expression of the FMR and outlying gene. Expression levels identical to those of the parent strain, given the limits of the instrument, were found in the 11168-O *nssR*-FLAG strain for the genes between *pseI* (Cj1317) and *pseE* (Cj1337) inclusive (data not shown). As the expression of Cj0466 (*nssR*) and the immediate downstream gene Cj0467 had not been previously analysed in the wild-type the expression of these genes was examined in 11168-O WT and 11168-O *nssR*-FLAG. Figure 3.12 shows representative expression levels of Cj0466 and Cj0467 in both strains, compared to the internal control gene *gyrA*. This indicates that *nssR* and Cj0467 are expressed at the same level and suggests that the replacement of the wild-type *nssR* gene with the *nssR*-FLAG construct has not adversely affected expression of this gene. This suggests that the ChIP-chip work can be carried out using this strain with reasonable confidence that any results generated are not artefactual.



**Figure 3.12. Quantitative real-time PCR assays of Cj0446 and Cj0467 gene expression in 11168-O WT and 11168-O *nssR*-FLAG.** The relative change in gene expression compared to the reference gene (*gyrA*) is shown, calculated using the  $2^{-\Delta\Delta C_T}$  method. The error bars represent the range determined using the  $2^{-\Delta\Delta C_T}$  calculation, with  $\Delta\Delta C_T + S$  and  $\Delta\Delta C_T - S$  where  $S$  = the standard deviation of the value for  $\Delta\Delta C_T$ . These results represent a data set from one culture; the results were reproducible using separate RNA extracts from independent cultures.

#### 3.3.6.4 Development of the chromatin immunoprecipitation method

As chromatin immunoprecipitation from *Campylobacter* has not been previously reported, the method used here and detailed in section 3.2.12 was adapted from that used by Grainger *et al.* (2005). Alterations to increase the amount of quality of DNA isolated were made, including an increase in the number and stringency of the washing steps during immunoprecipitation to take into account the fact that a greater volume of more concentrated chromatin was needed for a sufficient amount of DNA to be isolated. To increase the stringency of the final wash buffer NaCl was added; LiCl was also tested but was found to produce conditions which were too stringent, as no DNA was isolated. An elution buffer containing NaHCO<sub>3</sub> was also substituted for Tris/EDTA/SDS buffer as it increased the amount of DNA eluted (Cho *et al.*, 2008). The conditions used to reverse the DNA-protein crosslink induced by formaldehyde were also increased to an overnight incubation at 65°C to decrease the amount of protein remaining on the DNA prior to the protease digestion. Proteinase K was substituted for the Pronase used by Grainger *et al.* (2005) as preliminary PCR experiments showed that it removed more protein from the immunoprecipitated DNA.

#### 3.3.6.5 Failure of direct labelling

In order to identify the genes present in the DNA isolated by ChIP and so identify the location of NssR binding, it was necessary to first label the DNA with the appropriate CyDye. A BioPrime Array CGH Genomic Labelling System was used to attempt to label the DNA; this system has previously been used to successfully label ChIP DNA for use on microarrays (C. Constantinidou, personal communication). The system incorporates the fluorescently labelled nucleotides into the DNA during a polymerisation reaction catalysed by the exo-Klenow enzyme and using random

octamers, which should result in an approximately 10-fold amplification of DNA; the resulting DNA is then purified from the reaction prior to use in microarray hybridisation.

Unfortunately, all attempts at the direct labelling of ChIP DNA isolated during this work failed, despite repeated attempts at troubleshooting which included varying the amounts of ChIP DNA added to the reactions and the amount of exo-Klenow DNA polymerase used. Extended incubations of protease with the ChIP DNA were also used in an attempt to remove any protein remaining on the DNA from the initial DNA-protein cross-linking with formaldehyde. If any protein had resisted the protease digestion and heating used to reverse the cross-links during the ChIP protocol, it could interfere with the progress of the exo-Klenow DNA polymerase along the DNA strand, causing premature termination of polymerisation and resulting in failure of the reaction. Unfortunately these attempts also ended in failure and it was not possible to generate fluorescently labelled DNA suitable for microarray hybridisation before the end of the project.

#### 3.3.6.6 Analysis of immunoprecipitated DNA by PCR

Due to the failure of the direct labelling procedure it proved impossible to use high-density microarrays to obtain a genome-wide picture of NssR binding as was originally planned. However, the process of troubleshooting the direct labelling reaction also identified an alternative, albeit more limited, method for the analysis of the DNA isolated by chromatin immunoprecipitation. It was found that PCR with a *Taq* DNA polymerase, as opposed to the exo-klenow enzyme used for the direct labelling reaction, was successful. Hence the ChIP DNA was instead analysed by PCR and examined for the presence of a defined set of genes, including those of the FMR and surrounding

genes together with *nssR* itself and *cgb*, a single domain globin known to be regulated by NssR under nitrosative stress conditions. The flagellin genes were also tested for but due to the high sequence similarity exhibited by these genes it was only possible to test for both *flaA* and *flaB* together, in Table 3.10 below these are listed as '*fla*'; as Cj1318 and Cj1335/6 are direct repeats it was only possible to test for these genes in combination. The AB<sup>+</sup> (ChIP carried in the presence of the appropriate antibody), AB<sup>-</sup> (ChIP carried out with the antibody substituted by water) and total chromatin fractions were tested from both the 11168-O WT DNA immunoprecipitated using the polyclonal NssR antibody and the 11168-O *nssR*-FLAG DNA immunoprecipitated using the monoclonal FLAG antibody. The results are given in Table 3.11, each set of immunoprecipitations were carried four times and all samples were tested by PCR, the results were consistent for all comparative samples.

The results were consistent between both immunoprecipitation methods used, indicating that adding the FLAG-tag to the coding sequence of the *nssR* gene in the 11168-O *nssR*-FLAG strain had no observable effect on the action of the NssR protein. The fact that the results were consistent across all samples indicates that the immunoprecipitation method developed here is capable of consistently isolating DNA.

**Table 3.11. PCR testing of chromatin immunoprecipitated DNA.** ‘+’ indicates that the PCR generated an amplicon of the correct size for that sample, ‘-’ indicates that no PCR amplicon was produced. ‘AB<sup>+</sup>’: ChIP carried in the presence of the appropriate antibody; ‘AB<sup>-</sup>’: ChIP carried out where the appropriate antibody was substituted for water and ‘Total’: chromatin which has not been used for immunoprecipitation and was isolated from the appropriate *C. jejuni* culture.

Gene	11168-O WT			11168-O <i>nssR</i> -FLAG		
	AB <sup>+</sup>	AB <sup>-</sup>	Total	AB <sup>+</sup>	AB <sup>-</sup>	Total
<i>pseI</i> (Cj1317)	-	-	+	-	-	+
Cj1318/36	+	-	+	+	-	+
Cj1319	+	-	+	+	-	+
Cj1320	-	-	+	-	-	+
Cj1321	-	-	+	-	-	+
Cj1322	-	-	+	-	-	+
Cj1323	-	-	+	-	-	+
<i>ptmG</i> (Cj1324)	+	-	+	+	-	+
<i>ptmH</i> (Cj1325/6)	-	-	+	-	-	+
<i>ptmC</i> (Cj1327)	-	-	+	-	-	+
<i>ptmD</i> (Cj1328)	-	-	+	-	-	+
<i>ptmE</i> (Cj1329)	-	-	+	-	-	+
<i>ptmF</i> (Cj1330)	-	-	+	-	-	+
<i>ptmB</i> (Cj1331)	+	-	+	+	-	+
<i>ptmA</i> (Cj1332)	-	-	+	-	-	+
<i>pseD</i> (Cj1333)	-	-	+	-	-	+
Cj1334	+	-	+	+	-	+
<i>pseE</i> (Cj1337)	-	-	+	-	-	+
<i>fla</i> (incorporating <i>flaA</i> & <i>flaB</i> )	-	-	+	-	-	+
<i>nssR</i>	+	-	+	+	-	+
<i>cgb</i>	+	-	+	+	-	+

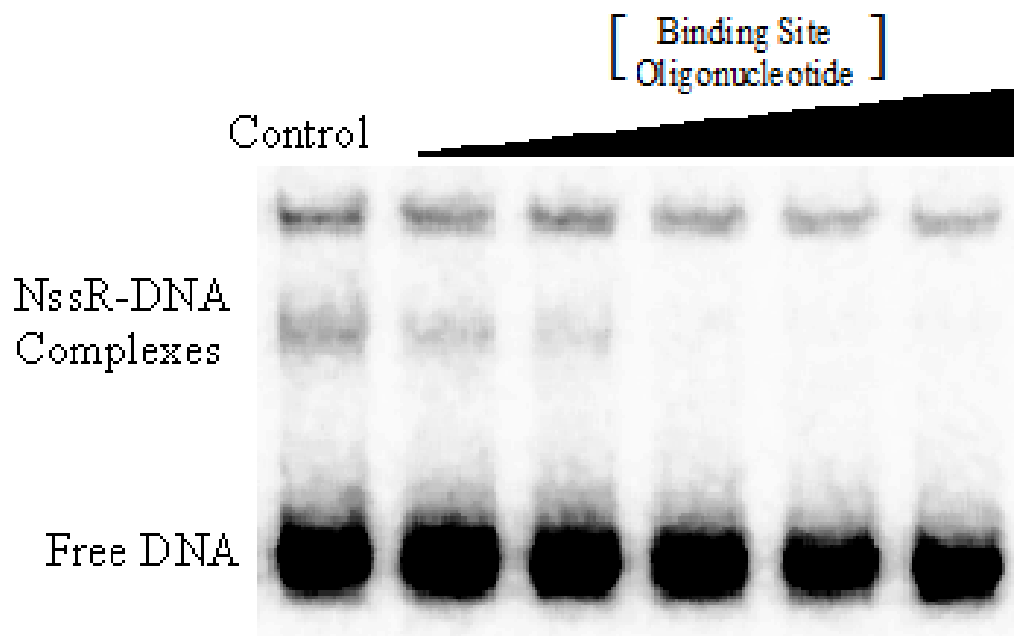
### 3.3.7 NssR binds DNA *in vitro*

An electrophoretic mobility shift assay was carried out using purified, His•tagged NssR protein and P<sup>32</sup>–labelled DNA fragments amplified from the promoter region of the *cgb* gene, which contains the consensus NssR binding site under nitrosative stress conditions, TTAAC-N<sub>4</sub>-GTTAA. The retardation of the movement of radio-labelled *cgb* DNA in the presence of NssR, compared to the unimpeded movement of the radio-labelled *cgb* DNA alone, indicates that NssR is able to bind *in vitro* to this DNA; a representative gel image is shown in Figure 3.13. When an increasing concentration of unlabelled oligonucleotides carrying the NssR binding site sequence (TTAAC-N<sub>4</sub>-GTTAA) was added to reactions containing 3.2μM NssR and the same radio-labelled DNA as used above, the DNA-NssR band disappeared as greater concentrations of the unlabelled oligonucleotide were added, Figure 3.14. This result also suggests that the NssR protein is binding to DNA containing the NssR consensus binding sequence. This sequence was deduced by examining the promoter regions of genes induced by NssR under conditions where nitrosative stress was present. Non-specific binding of NssR to DNA would not have been detected by this method as unlabelled herring sperm DNA was present in all reactions in a large excess. These results together suggest that NssR is able to bind, *in vitro* and in the absence of externally applied nitrosative stress, to the consensus binding sequence known to be used under conditions where nitrosative stress has been applied; whether this binding also occurs *in vivo* is unknown.



**Figure 3.13 NssR binds DNA containing the NssR binding motif in the absence of externally applied NO stress.** Electrophoretic mobility shift assay showing binding of purified NssR protein to radio-labelled DNA fragments from the *cgb* promoter region and the resulting retardation of movement of the DNA, in comparison to unbound, free DNA. The DNA fragments were incubated with 0.32, 0.16, 0.08, 0.04, 0.02 or 0 $\mu$ M (control) NssR as indicated.





**Figure 3.14 Loss of NssR binding upon addition of an oligonucleotide containing the NssR binding motif.** An electrophoretic mobility shift assay showing loss of binding of purified NssR protein to radio-labelled DNA fragments from the *cgb* promoter region when an oligonucleotide containing the NssR binding sequence TTAAC-N4-GTTAA was added in increasing concentrations. The DNA fragments were incubated with 0.32 $\mu$ M NssR and the concentrations of the oligonucleotide added were 0 $\mu$ M (control), 0.10 $\mu$ M, 0.25 $\mu$ M, 0.50 $\mu$ M, 1.0 $\mu$ M, 10.0 $\mu$ M respectively.

### 3.4 Discussion

The overall aim of this part of the study was to determine the effects that NssR has on the small subset of flagellin glycosylation genes that constitute that FMR. A mutant in *nssR* constructed in the 11168-O strain background of *C. jejuni* 11168 did not express the genes of the FMR. However, a similar mutant constructed in the 11168-GS background showed no loss of expression of the FMR, indicating that NssR is not involved in the regulation of these genes in this strain. Hence, the work here was focused on the activity of NssR in strains of the 11168-O background.

**Table 3.12 Analysis of expression of the FMR of *C.jejuni*, by quantitative RT-PCR, qualitative qPCR and chromatin immunoprecipitation.**

Gene	Transcript continuity tested by RT-PCR <sup>a</sup>	Expression tested by qPCR <sup>b</sup>	Tested by 5'RACE as potential transcript start site	NssR assessed by ChIP <sup>c</sup>
<i>pseI</i> (Cj1317)	+	+	-	-
Cj1318 <sup>d</sup>	+	+	+	+
Cj1319	Cj1320	+	+	+
Cj1320	+	+	-	-
Cj1321	+	+	-	-
Cj1322	-	-	-	-
Cj1323	-	-	-	-
<i>ptmG</i> (Cj1324)	+	+	+	+
<i>ptmH</i> (Cj1325/6)	<i>ptmC</i>	+	+	-
<i>ptmC</i> (Cj1327)	+	+	-	-
<i>ptmD</i> (Cj1328)	<i>ptmE</i>	+	-	-
<i>ptmE</i> (Cj1329)	+	+	-	-
<i>ptmF</i> (Cj1330)	-	-	-	-
<i>ptmB</i> (Cj1331)	<i>pseD</i>	+	+	+
<i>ptmA</i> (Cj1332)	<i>pseD</i>	+	-	-
<i>pseD</i> (Cj1333)	+	+	-	-
Cj1334	+	+	-	+
Cj1335/6 <sup>d</sup>	+	+	-	+
<i>pseE</i> (Cj1337)	+	+	-	-

**a:** transcript '+' present, '-' absent or 'Cj1340' - transcript contains all genes up to and including Cj1340.

**b:** expression '+' present, '-' absent.

**c:** NssR binding '+' present, '-' absent.

**d:** Cj1318 and Cj1336 are directly repeated genes, hence it is not possible to distinguish between these genes.

### 3.4.1 Transcriptional organisation of the flagellar modification region.

The first stage of the analysis of NssR activity in the FMR involved deducing the transcriptional organisation of the FMR. To that end, transcripts across the FMR were mapped by qualitative reverse-transcription PCR (RT-PCR) to determine the presence and extent of any transcripts in this region and to discover whether this region was transcribed discretely or together with the surrounding genes. Quantitative qPCR was also carried out to examine the levels of gene expression in this region, relative to the control gene *gyrA*.

These combined analyses indicated that the FMR is transcribed independently to the surrounding genes *pseI* (Cj1317) and *pseE* (Cj1337). Also evident was the fact that the region was expressed, not as a single long transcript from one promoter, but in several transcripts, some containing multiple genes, others only a single gene. The data from the RT-PCR and qPCR analyses are shown in table 3.12 together with the data gathered from both ChIP approaches, this data is generally in agreement upon the genes constituting each transcript, although there was a level of variation which can be explained by the limitations of each method. Transcriptional organisation was inferred from the qPCR data by comparing the expression levels of neighbouring genes; hence a gene which was expressed at a level less than the upstream gene would appear to form part of the preceding transcript, a gene which appeared to be expressed at a level greater than the preceding gene could be said to form a new transcript. The RT-PCR approach was limited by the activity of the reverse-transcriptase used to convert mRNA into cDNA, limiting the length of transcript which could be detected, and generally only transcripts less than 3kb were searched for. The combined results suggest that the FMR is expressed on different transcripts comprising 1) Cj1319, Cj1320 and Cj1321, 2)

*ptmG*, 3) *ptmH* and *ptmC*, 4) *ptmD*, *ptmE* and *ptmF*, 5) *ptmB*, *ptmA* and *pseD* and 6) Cj1334. It was impossible to analyse the expression levels or transcription of Cj1318 and Cj1335/6 independently as these genes are direct repeats and hence are identical in sequence.

No evidence of the transcription of Cj1322 or Cj1323 was observed, whether this was due to the actual level of expression being below the level of detection of the qPCR instrument or whether these genes are in fact not expressed in 11168-O WT it is impossible to determine. BLAST analyses of the predicted amino acid sequence of Cj1322 and Cj1323 showed no homology to any bacterial proteins. Expression of *ptmF*, which is thought to have a role in legionaminic acid biosynthesis (Logan *et al.*, 2002), was confirmed by qPCR, although the expression was at a very low level, which could explain why its expression was not detected by RT-PCR transcript mapping.

Analysis of the FMR by qPCR in the 11168-O 0466<sup>-</sup> mutant indicated that transcription of the FMR was abrogated in this strain, supporting the conclusion that NssR is involved in FMR regulation. The surrounding genes, *pseI* and *pseE*, were expressed at levels comparable to the wild-type, indicating that these genes are unlikely to be subject to regulation by NssR.

### 3.4.2 Location of potential promoter sequences in the FMR

Having determined the likely transcriptional organisation of the FMR, it was possible to determine that FMR transcription does not originate from a single promoter at the start of the region, and to locate potential transcriptional start points within the FMR. Identification of transcript start points would, in addition to confirming the transcriptional organisation of the FMR, allow a search for the associated promoter sequences to be targeted. The combined data from the qualitative RT-PCR, quantitative

qPCR and combined ChIP approaches, shown in Table 3.12, is in largely in agreement upon the location and constituent genes of each transcript; the locations of potential transcript start points was inferred from his data. As the FMR was transcribed separately to the surrounding genes, it is likely that a promoter would be located in the first genes of the FMR. As it was difficult to assess the transcription of Cj1318, the upstream sequences of both it and Cj1319 were examined using 5' RACE. Also examined were the upstream regions of *ptmG*, *ptmH*, *ptmB* as these genes were located at the start of the transcripts indentified above. No transcript start points for any so these genes were identified, despite multiple attempts. This failure is likely to be due to the fact that the primers designed to amplify the 5'-regions of these transcripts were incorrectly located. Primer extension is another popular technique for the exact identification of transcript start points, unfortunately this method also relies a correctly located primer to be successful. As all previous attempts to design primers for this purpose had been unsuccessful, the identification of transcript start points by primer extension was not attempted and it proved impossible to locate the transcriptional start points of the FMR.

### 3.4.3 NssR binds DNA *in vitro*

NssR is the only member of the Crp-Fnr super-family of DNA binding proteins present in *C. jejuni* NCTC 11168. The promoter regions of genes regulated by members of the Crp-Fnr super-family generally contain distinctive recognition motifs (Korner *et al.*, 2003). One such motif, with a consensus sequence of TTAAC-N<sub>4</sub>-GTAA, was identified in the promoter regions of those genes in *C. jejuni* NCTC 11168 regulated by NssR, in the presence of externally applied nitrosative (NO) stress. These genes include the single-domain globin Cgb, which is involved in the scavenging and detoxification

response to NO stress (Elvers *et al.*, 2004), and several other genes of uncertain function.

The binding of NssR to DNA at the motif given above in the presence of nitrosative stress has been established (Elvers *et al.*, 2005). As part of this work, electrophoretic mobility shift assays were used to test for NssR binding in the absence of NO stress. Binding of NssR to radio-labelled DNA containing the consensus binding motif, in the absence of external NO stress was observed. The application of increasing concentrations of an unlabelled oligonucleotide, containing the consensus binding sequence, to the mixture of purified NssR and radiolabelled *cgb* DNA, produced a gradual decrease in the level, and eventually removal, of NssR binding. A large excess of salmon-sperm DNA was also applied to all reactions to ensure that non-specific binding would not produce a false positive result. Together, these results indicate that, at least *in vitro*, NssR is able to bind DNA at the consensus binding sequence, without the application of externally applied NO stress. Whether this binding also occurs *in vivo* can not be inferred from this data.

If NssR is able to bind DNA and the sequence given above and if it were to act directly upon the FMR, binding motifs may also be visible in the promoter regions of FMR genes. However, a search of the entire FMR DNA sequence for the consensus binding sequence identified no motifs with zero or one mismatches to the consensus and too many motifs with two or three mismatches to identify any specific motifs which could form part of a promoter sequence.

### 3.4.4 Chromatin immunoprecipitation (ChIP) to assess NssR binding

A ChIP method for the isolation of those genomic DNA fragments to which NssR binds *in vivo* in the 11168-O WT and a strain constructed in the 11168-O background in which a FLAG-NssR fusion replaced wild-type NssR, was successfully developed. Initially the aim of this part of the work was to produce a genome-wide ‘map’ of NssR binding by applying the DNA isolated by ChIP to a high-density DNA microarray (ChIP-chip) and identifying all genes to which NssR bound in the growth condition used. This ‘map’ could then be used to identify any other genes regulated by NssR and to determine whether the action of NssR upon the genes of the FMR was direct, acting upon the genes themselves, or indirect, involving the modulation of expression of a second, unknown, regulatory gene.

However, the failure of the DNA isolated by ChIP to label with the Cy-dyes necessary for detection on a microarray, meant that it was not possible, in the time available, to use the microarray to assess genome-wide NssR binding. Instead, the presence of a small subsection of genes, including those of the FMR, in the DNA isolated was tested for by PCR. The results generated from both ChIP approaches were in agreement and indicated that NssR bound to DNA in which the Cj1318/36, Cj1319, *ptmG*, *ptmB* and Cj1334 genes were present. As the DNA isolated from ChIP was sonicated to produce fragments of between approximately 500bp and 1kb it is possible that some DNA fragments were outside of this range and contained DNA from neighbouring genes. Hence it is possible that NssR binds only to one location where consecutive genes have been identified, for example in Cj1318 or Cj1319 and Cj1334 or Cj1335/6 not in both. Also, the identical sequence of Cj1318 and Cj1335/6 means that it is impossible to determine whether NssR binds to a sequence in both or only in one of these genes.



The failure of direct labelling with Cy-dyes was potentially due to the presence of protein, NssR or NssR-antibody, cross-linked to the DNA. Although several different methods of de-cross-linking and protease digestion were attempted, none removed the protein from the DNA to a point where the Exo-Klenow fragment polymerase was able to amplify DNA and allow labelling. The ability of the *taq* enzyme to amplify ChIP DNA may simply be due to the robustness of the enzyme in comparison to Exo-Klenow. The retention of DNA on ChIP DNA is an acknowledged problem with this technique (Cho *et al.*, 2008).

#### 3.4.5 Variation of homopolymeric tracts

To assess whether those FMR genes which contain homopolymeric sequences, Cj1318 and Cj1335/6, could be subject to phase variation by slipped strand mispairing, the homopolymeric tract located within Cj1318 was amplified from several separate cultures of 11168-O WT and 11168-GS WT and sequenced. None of the sequences generated from 11168-GS WT DNA showed any variation in the number of residues in the homopolymeric tract. Whilst the majority of 11168-O WT sequences examined also showed no variation in the sequence of this tract, one sequence showed variation between 10 and 11 G-residues; the population was divided approximately equally between the two states. This concurs with the findings of Parkhill *et al.* (2000) who, during the *C. jejuni* NCTC 11168 genome sequencing project, identified variation in this tract of between 10 and 11 G-residues, with the majority of the population being in the phase ‘off’ state with 10 G-residues. In order for the Cj1318 gene to be in the phase ‘on’ state the tract must contain 11 G-residues.

Cj1318 (*mafI*) is part of the *maf* (motility accessory factor) family of genes, which share considerable homology; some members of this group are involved in the

attachment (*pseD/maf2*) or assembly (*pseE/maf5*) of flagellar glycans. The exact function of Cj1318 is unknown, however, Karlyshev *et al.* (2002) demonstrated that a functional Cj1318 gene, with a homopolymeric tract containing 11 G-residues, could partially restore motility in an otherwise non-motile Cj1337 (*maf5*) mutant. Since this work was carried out, a homolog of Cj1337 (*maf5*) in *C. jejuni* 81-176 was found to have a role in the assembly or transfer of pseudaminic acid derived glycans to flagellin and was annotated *pseE* (Guerry *et al.*, 2006). As a functional Cj1318 protein was able to partially restore the motility of a Cj1337 mutant, it is possible that Cj1318 may at least partially functionally substitute PseE and hence potentially have a similar function.

#### 3.4.6 Influence of NssR on the FMR

Despite the fact that a complete map of NssR binding was not generated, the results generated by ChIP agree well with the interpretation of the transcriptional organisation of the FMR derived from RT-PCR and qPCR, which suggested that the FMR could be expressed in approximately six transcripts originating from Cj1319, *ptmG* (Cj1324), *ptmH* (Cj1325/6), *ptmD* (Cj1328), *ptmB* (Cj1331) and Cj1334. The ChIP data also supports the evidence of Section 3.4.3 that indicates that NssR binds DNA in the absence of external NO stress, although whether the NssR binding motif is the binding sequence used *in vivo* can not be determined. Together, these results suggest that NssR is able to function in the absence of externally applied nitrosative stress and is likely to bind directly to certain undefined sequences, probably located in the promoter regions of Cj1318/36, Cj1319, *ptmG*, *ptmB* and Cj1334, to modulate the expression of these genes. As the transcript start points of these genes could not be identified it also proved to be impossible to identify a possible binding motif for NssR

under these conditions, whether the NssR binding motif identified by Elvers *et al.* (2005) or a novel sequence.

It is also possible that NssR is not the only regulatory protein active in the FMR, as several genes, particularly *ptmH* and *ptmD*, which were not indentified by ChIP as being binding locations for NssR, show evidence of being transcribed independently of upstream genes. Carrillo *et al.* (2004) also identified  $\sigma^{28}$  promoter sequences upstream of *ptmA* and *ptmB*, which indicates that the flagellar associated  $\sigma$ -factor may also be involved in regulation of flagellar glycosylation.

### 3.4.7 Future work

Whilst the failure of direct labelling meant that it was impossible to generate a genome wide map of NssR binding, the fact that DNA, corresponding to genes known to be directly regulated by NssR (*cgb* and *nssR*), as well as to genes not previously identified as being directly regulated by NssR (FMR genes), was isolated using the ChIP method developed as part of this work demonstrates the effectiveness of this method. The high throughput DNA sequencing technologies developed in recent years, such as the Illumina platform and the 454 technology of Roche, are capable of generating at least thousands of sequences from one run (Hall, 2007). The use of these high-throughput DNA sequencing technologies to sequence DNA isolated by ChIP (ChIP-seq) using anti-NssR antibodies, would allow a genome-wide map of NssR binding to be generated without the use of a microarray. The transcriptome of the *nssR* mutant could also be examined using RNA-seq, another high-throughput sequencing technique able to identify all of the mRNA transcripts present in a sample. The combination of NssR binding location mapping by ChIP-seq and transcriptome sequencing by RNA-seq would provide a very complete picture of the genome-wide activity of NssR.

# Chapter IV: Motility and biofilm formation in *C. jejuni* 11168

## 4.1 Introduction

### 4.1.1 Motility in *Campylobacter*

The characteristic rapid, darting motility of *Campylobacter* is mediated by a single polar unsheathed flagellum located at one or both ends of the cell (monotrichous or amphitrichous, respectively). This flagellar mediated motility is an essential part of the pathogenesis of *Campylobacter*, allowing cells to translocate through the viscous mucus of the intestine to colonise and establish an infection. Flagellar motility is also involved in the attachment to and invasion of cells, as shown by Wassenaar *et al.* (1991) and Grant *et al.* (1993) using polarised intestinal cell monolayers.

The flagella of *Campylobacter jejuni* are of the complex type, being composed of two species of flagellin monomer, as opposed to the single flagellin seen in *E. coli*. FlaA is the major flagellin in *Campylobacter*, expression of which is dependent on a  $\sigma^{28}$  promoter, while the gene encoding the minor flagellin, FlaB, is located downstream of FlaA and is dependent on a  $\sigma^{54}$  promoter (Guerry *et al.*, 1990; Nuijten *et al.*, 1990). The two flagellins show considerable homology, approximately 95% in the sequenced strain of *C. jejuni* NCTC 11168 (11168-GS WT), with the majority of the variation occurring in the central region of the sequence. Mutants in *flaA* are poorly motile with short, truncated flagella, whilst mutants in *flaB* possess near normal flagella and motility but in certain experiments show a decrease in pathogenic potential (Guerry *et al.*, 1991; Wassenaar *et al.*, 1993). It has been suggested, by Kostrzynska *et al.* (1991) that in the closely related bacterium *Helicobacter pylori* FlaB forms the proximal part of the

flagellum, while FlaA forms the distal part. However, as the two proteins in *C. jejuni* are antigenically indistinguishable, it is not possible to ascertain whether this is the case in this species. Flagellins in *Campylobacter* are also glycosylated, a modification which could have a role in processes as diverse as flagellar biogenesis and immune system or bacteriophage evasion. The exact extent of glycosylation in *C. jejuni* NCTC 11168 is unknown, but in the closely related strain *C. jejuni* 81-176, approximately 10% of the flagellar mass consists of carbohydrates (Thibault *et al.*, 2001). Whilst the exact function(s) of flagellin glycosylation remain unclear, it is known that a variety of mutants in *C. jejuni*, where the flagellin lacks glycans or is glycosylated differently to the wild-type, are unable to assemble a full-length flagellum. Certain mutants have also been seen to accumulate unglycosylated flagellin monomers intracellularly (Goon *et al.*, 2003) and other mutants do synthesise flagella but are non-motile.

*C. jejuni* strains also show the ability to move, reversibly and apparently spontaneously, between flagellate and aflagellate phenotypes. This indicates that *C. jejuni* has the capacity to modulate motility by variable expression of the flagella. Whether the variable expression of flagellin glycosylation genes is involved in this process is unclear (Caldwell *et al.*, 1985). The sequenced strain of *C. jejuni* NCTC 11168 (11168-GS WT) appears to be subject to some kind of flagellar phase switching, as many stocks of this strain are non-motile but still retain intact full-length flagella, including that used in this study. Motility of these stocks can be selected for *in vitro* using soft 'motility' agar as demonstrated by Karlyshev *et al.* (2002). This ability to modulate flagellar expression, function and/or flagellin glycosylation is understandable in light of the energetic cost of flagellar expression and the need for most pathogenic bacteria to adapt to the environmental changes encountered during the infection of a

host and exposure to the external environment, for those bacteria which are transmitted by the food chain.

The 11168-GS WT strain used in this study exhibits full-length flagella but is almost always non-motile; only rarely will an individual culture exhibit some motility, when approximately 50% of the cells move in a manner reminiscent of the 11168-O WT (Personal observation). The 11168-GS 0466<sup>-</sup> and 11168-GS 0466C strains are both non-motile and exhibit full-length flagella. The 11168-O WT and 11168-O 0466C strains always exhibit the rapid darting motility characteristic of *Campylobacter*. However, the 11168-O 0466<sup>-</sup> mutant does not consistently have full-length flagella when examined by electron microscopy and whilst the cells are still motile, they do not demonstrate the same type of movement as the parental strain. As the type of motility defect shown by the 11168-O 0466<sup>-</sup> mutant has not previously been documented, this defective motility will be examined and quantified, for comparison with the wild-type.

To ensure that the most suitable method(s) are used to examine motility several methods previously used to analyse and record bacterial motility will be tested and if necessary adapted and developed, to produce a method which allows repeatable and reliable quantification of bacterial motility and that yields results which can be easily presented. To aid in the development of a suitable method the motility of the other strains used in this study will also be examined, particularly that of the 11168-O WT, as due to its speed, it is likely to be the most challenging strain to analyse. The methods to be tested include the Hobson BacTracker imaging system which had been used by Karim *et al.* (1998) to measure and compare the motility of *H. pylori*, *C. jejuni* and *E. coli*. The second method is the ImageJ based video microscopy and manual tracking system which has been used to examine the motility of  $\Delta flhF$  mutants in *Pseudomonas aeruginosa* and to produce images showing the paths of movements of individual

bacterial cells (Murray and Kazmierczak, 2006). The final method used involves growth of the strains on soft 'motility' agar, as demonstrated by Karlyshev *et al.* (2002) and used in the laboratory to isolate motile revertants from previously non-motile mutants.

#### 4.1.2 Biofilm formation

Biofilms are multi-layered complex bacterial ecosystems encased in a polysaccharide matrix; they exist in many different ecological niches and are important considerations in many different industries, particularly those involved in food production, water treatment and also in medicine. Monospecies biofilms are of clinical importance and can be formed by several human pathogens; whilst *C. jejuni* has not been identified as one of these species, it has been found as part of certain multi-species biofilms (Trachoo *et al.*, 2002). Biofilms can confer protection against a variety of stresses including desiccation, nutrient depletion and antibiotic exposure, hence the formation of or inclusion in biofilms may aid the survival of *Campylobacter* in the environment. Flagella appear to be involved in the initial attachment phase of biofilm formation as aflagellate mutants do not attach to other cells or surfaces (Joshua *et al.*, 2006; Kirov *et al.*, 2004).

Autoagglutination, which has been demonstrated in *Campylobacter* (Golden and Acheson, 2002), involves non-specific reactions which lead to clumping of bacterial cells, although it is unclear whether this has any role in biofilm formation or growth (Guerry *et al.*, 2006). Autoagglutination in *Campylobacter* is at least partly mediated by flagella and it is possible that flagellar glycans have some involvement in this process (Guerry *et al.*, 2006). Several transposon mutants in genes thought to be involved in flagellin glycosylation have been found to be deficient in autoagglutination (Golden and Acheson, 2002).



Howard *et al.* (2009) have identified a livestock clade associated glycosylation island (Cj1321-Cj1325/6), which has a role in maintaining the ability of *Campylobacter* to colonise chickens. Deletion of this island, and particularly the Cj1324 gene, also annotated as *ptmG*, also caused an alteration in cell surface charge, a decrease in the ability of this strain to autoagglutinate and form biofilms, together with a loss of LegAm from flagellin. This flagellar glycan loss is thought to be responsible for the decrease in chicken colonisation seen in these mutants; both the complete island and Cj1324 mutants remained motile and formed wild-type length flagella. This genetic island forms part of the FMR down-regulated in the 11168-O 0466<sup>-</sup> mutant; hence it is possible that a decrease in autoagglutination and biofilm formation by the 11168-O 0466<sup>-</sup> mutant could be observed.

The formation of biofilms on glass surfaces by *C. jejuni* has been clearly demonstrated by Joshua *et al.* (2006). Autoagglutination in these strains has also been established and examined by Misawa and Blaser (2000). The ability of all *Campylobacter* strains used in this study to form biofilms on a glass surface, at the air-liquid interface of a culture or in the body of the liquid, which would be indistinguishable from autoagglutination occurring independently of biofilm formation, will be tested. Any differences identified in biofilm formation or autoagglutination between these strains may aid the characterisation of any differences in flagellin glycosylation between these strains.

## 4.2 Methods

### 4.2.1 Bacterial strains

A list of bacterial strains and their relevant characteristics is given in Table 2.3 (Section 2.3). All strains were grown under the culture conditions given in Section 2.4, unless otherwise stated.

### 4.2.2 Plate assay of motility

Motility assays of all strains from the 11168-O and 11168-GS backgrounds were carried out on soft agar plates made by combining Mueller-Hinton broth powder (Oxoid Basingstoke, UK) with 3.4g per litre of agar powder (Oxoid, Basingstoke, UK) to produce soft agar with a final agar concentration of 0.34% (w/v). An overnight broth culture of the strain to be tested was used to inoculate 10 ml MH broth to an OD<sub>600nm</sub> of 0.08; this culture was then grown with shaking until mid-log phase was reached at an OD<sub>600nm</sub> of approximately 0.3. The log phase culture (2 µl) was then either dropped onto the centre of a soft agar plate and allowed to soak in, stabbed into the body of the agar, taking care not to pierce the agar entirely and allow the inoculum to reach the plastic of the petri dish, or dropped onto a sterile 5 mm diameter filter paper disk placed onto the surface of the agar. Plates were then incubated microaerobically at 37°C, facing upwards to avoid disturbing the agar, for 48h and the diameter of any growth measured and photographed, using a Sony Cyber-shot 7.2 Mp camera, every 12h. After 24h incubation, samples were taken from the edges of bacterial growth on plates of the 11168-O WT and 11168-O 0466<sup>-</sup> strains for examination by electron microscopy to attempt to identify any difference in cell or flagellar morphology.

#### 4.2.3 Analysis of *C. jejuni* motility using the BacTracker

The swimming behaviours of all of the *C. jejuni* 11168-O strains were measured using the Hobson Bac Tracker imaging system (Hobson Tracking Systems, Sheffield, UK; Karim & Logan, 1998), a computerised tracking system capable of measuring the mean velocity of a culture of motile bacteria from video footage of their movement. Video footage of motile, log-phase bacteria was prepared and sent to Dr Q. N. Karim at Imperial College, London for analysis. Footage was prepared by diluting an overnight broth culture to an OD<sub>600nm</sub> of 0.1 and growing the culture microaerobically at 37°C for 4h to obtain a log phase culture with an OD<sub>600nm</sub> of between 0.2-0.3; all strains were also grown on agar plates for 40h. A drop of log phase culture, or 40h plate culture emulsified into MH broth, was prepared for filming as quickly as possible after leaving the incubator, to avoid any loss of motility due to exposure to atmospheric oxygen concentrations or temperature changes. Microslides (100µm; Camlab, Cambridge UK) were filled with the culture to be filmed by capillary action and sealed at each end using Critoseal (Camlab, Cambridge UK). Cultures were filmed using a Leitz Laborlux 12 microscope with a 40 x phase contrast objective. The camera used recorded directly to video tape, producing a field of view of approximately 25-35 cell lengths. The BacTracker software requires at least 2 minutes, and preferably 5 minutes, of recording, showing minimal drift, to make reliable measurements. Suitable recordings and repeats were made from each strain and growth condition.

#### 4.2.4 Analysis of motility by video microscopy and tracking.

The swimming behaviours, in MH broth, of wild-type and  $\Delta nssR$  bacteria from the *C. jejuni* 11168-O background were recorded then examined using the Image J software (Version 1.37, NIH; <http://rsb.info.nih.gov/ij/>) with the manual tracking plug-

in, a method of analysis adapted from Murray & Kazmierczak (2006). Cultures were prepared by diluting an overnight broth culture to an OD<sub>600nm</sub> of 0.1 and growing microaerobically at 37°C for approximately 4h to obtain a log phase culture with an OD<sub>600nm</sub> between 0.2- 0.3. A drop of log phase culture was prepared for filming as quickly as possible after leaving the incubator to avoid any loss of motility due to exposure to atmospheric oxygen conditions or temperature changes. Microslides (Camlab, Cambridge, UK) were used to attempt to avoid drift in the footage taken. Bacteria were observed by phase contrast microscopy with a Leitz Laborlux 12 microscope using a 40 x phase contrast objective, video clips were obtained with an Olympus SP-320 7.1 Mp camera. For each strain the movement, tracking on the centre of the cell, of six cells was followed for 60 consecutive images (2s). This was repeated for three separate films for each of three different biological replicates.

#### **4.2.5 Analysis of biofilm formation in *C. jejuni***

The ability of the strains of *C. jejuni* used in this study to form biofilms was tested. Growth by bacteria at the air-liquid interface at the surface of a broth culture produces a pellicle biofilm. Biofilms can also form when cells adhere to a surface or aggregate in liquid media to form flocs; the presence of any of these types of biofilm were recorded. A 10 ml overnight MH broth culture was diluted to an OD<sub>600nm</sub> of 0.1 and 3 ml of the dilution aliquoted into sterile 30 cm glass test tubes. At least 15 tubes for each strain were prepared and incubated microaerobically without shaking at 37°C for up to seven days. Two tubes of each strain were removed for examination every 24h. The presence of a pellicle and/or flocs was recorded and the OD<sub>600nm</sub> of the liquid phase was measured; the morphology of cells in the liquid phase and in any biofilms formed was examined microscopically. The test tubes were then washed gently with water,

stained for a least 5 minutes using 3 ml of 1% (w/v) crystal violet in water and washed a further three times with water to remove any loose debris, allowing closer examination of the attached biofilm.

## 4.3 Results

### 4.3.1 Plate assay of motility

#### 4.3.1.1 Reproducibility of the assay varies with inoculation method

The results obtained from plates inoculated using the stab method were more reproducible than those from plates where the culture had been dropped directly onto the surface of the agar or onto a filter paper disk. Motile bacteria were also more likely to spread into the agar on the plates inoculated using the stab method, rather than grow on the surface of the agar at the inoculation point only. Contamination was also slightly less common on stab inoculated plates versus plates inoculated using the drop method (5.9 % of total plates inoculated as opposed to 14.3%). Contamination was considerably more common on plates using the filter paper disks (30%) possibly as the plates had to been opened twice prior to incubation to apply both the paper disk and the inoculum. Consequently the stab method was used to inoculate the motility plates used in this study.

#### 4.3.1.2 Growth of strains on motility agar

The 11168-O background strains all grew and spread by growing into the body of the agar rather than on the surface of the plate, as no raised growth could be seen on the surface of the plate, suggesting that the cells were in fact moving through the soft agar itself. The *C. jejuni* 11168-O strain in which *nssR* (Cj0446) had been deleted,

11168-O 0466<sup>-</sup>, had covered significantly ( $P < 0.05$ ) less of the area of the soft agar plates after a 24h incubation when compared to both wild-type, 11168-O WT, and complemented mutant bacteria, 11168-O 0466C. However, after 36h the difference was less pronounced but still statistically significant ( $P < 0.05$ ) and by 48h all strains had covered the surface of the soft agar plates. Representative plates are shown in Figure 4.1 and the diameter of growth zone data summarised in Table 4.1.

#### 4.3.1.3 Examination of cells by electron microscopy

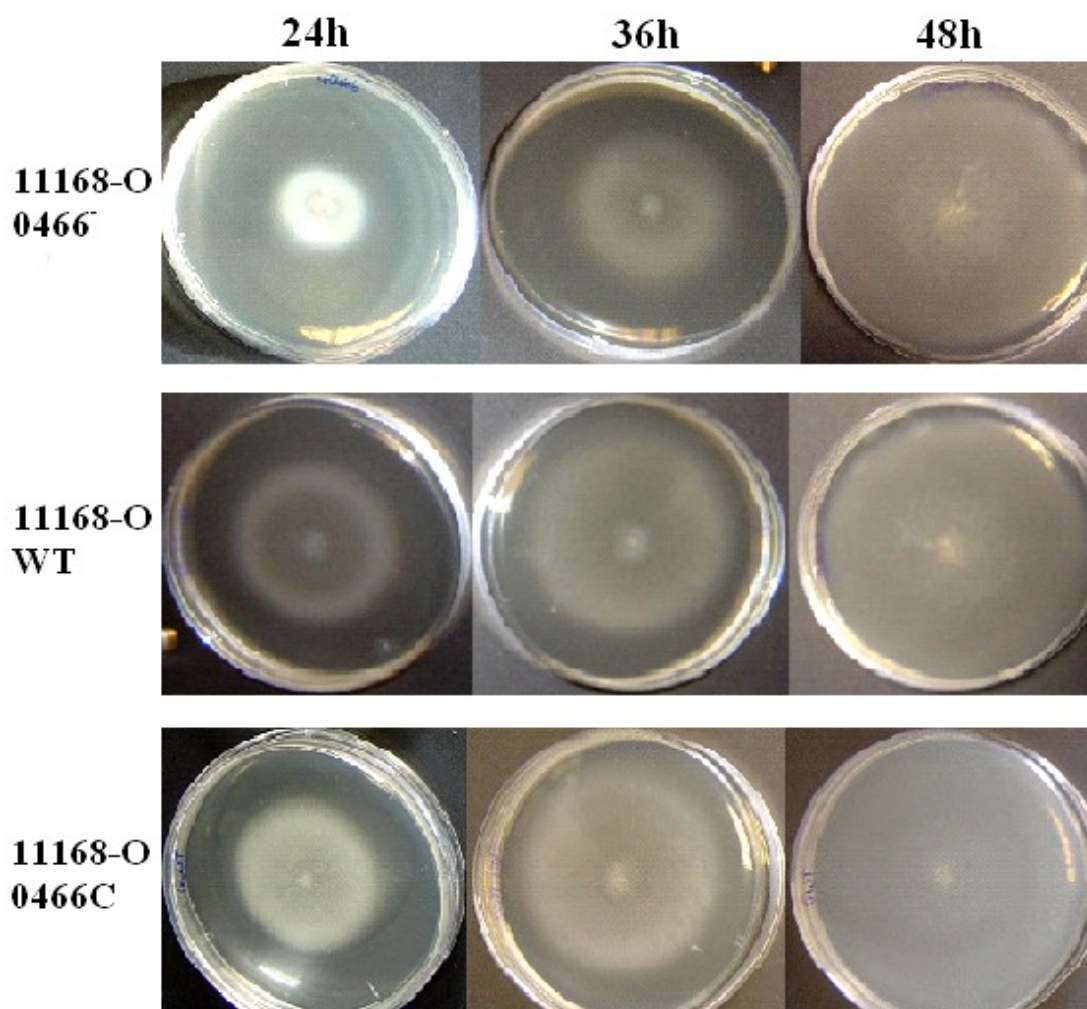
After 24h and 48h incubation on soft agar plates, samples were taken from the edges of the zones of growth on the 11168-O WT and 11168-O 0466<sup>-</sup> plates and prepared for electron microscopy to examine any differences in cell or flagellar morphology; representative images are shown in Figures 4.2 and 4.3. After 24h growth on soft agar cells of the 11168-O 0466<sup>-</sup> strain did not possess the normal full length wild-type flagella seen on the 11168-O WT. Many cells had short flagella and on some cells the presence of short, asymmetric flagella was apparent; after 48h growth cells from both strains had full length, wild-type flagella. The appearance of short, asymmetric flagella was not observed when the 11168-O 0466<sup>-</sup> strain was cultured on MH agar plates with a standard agar concentration for 24h, then examined by electron microscopy. When 11168-GS WT and 11168-GS 0466<sup>-</sup> cells were examined no differences in flagellar morphology were observed.

All of the 11168-GS background strains were repeatedly non-motile, although all of the strains from this background grew successfully on the agar. Growth remained confined to the central area of the plate in the area of inoculation and did not move across or into the agar. This method has also been used previously to select for motile derivatives of non-motile strains, such as the *C. jejuni* NCTC 11168 strain used by

Karlyshev *et al.* (2002) and also within our own laboratory to isolate motile revertants from previously non-motile mutants. These motile revertants appear on the agar as individual outgrowth sectors which grow from the central point of inoculation out towards the edge of the plate and which usually cover no more the one eighth of the surface of the plate. All plates inoculated with the non-motile 11168-GS background strains were examined for these outgrowths, but none were identified.

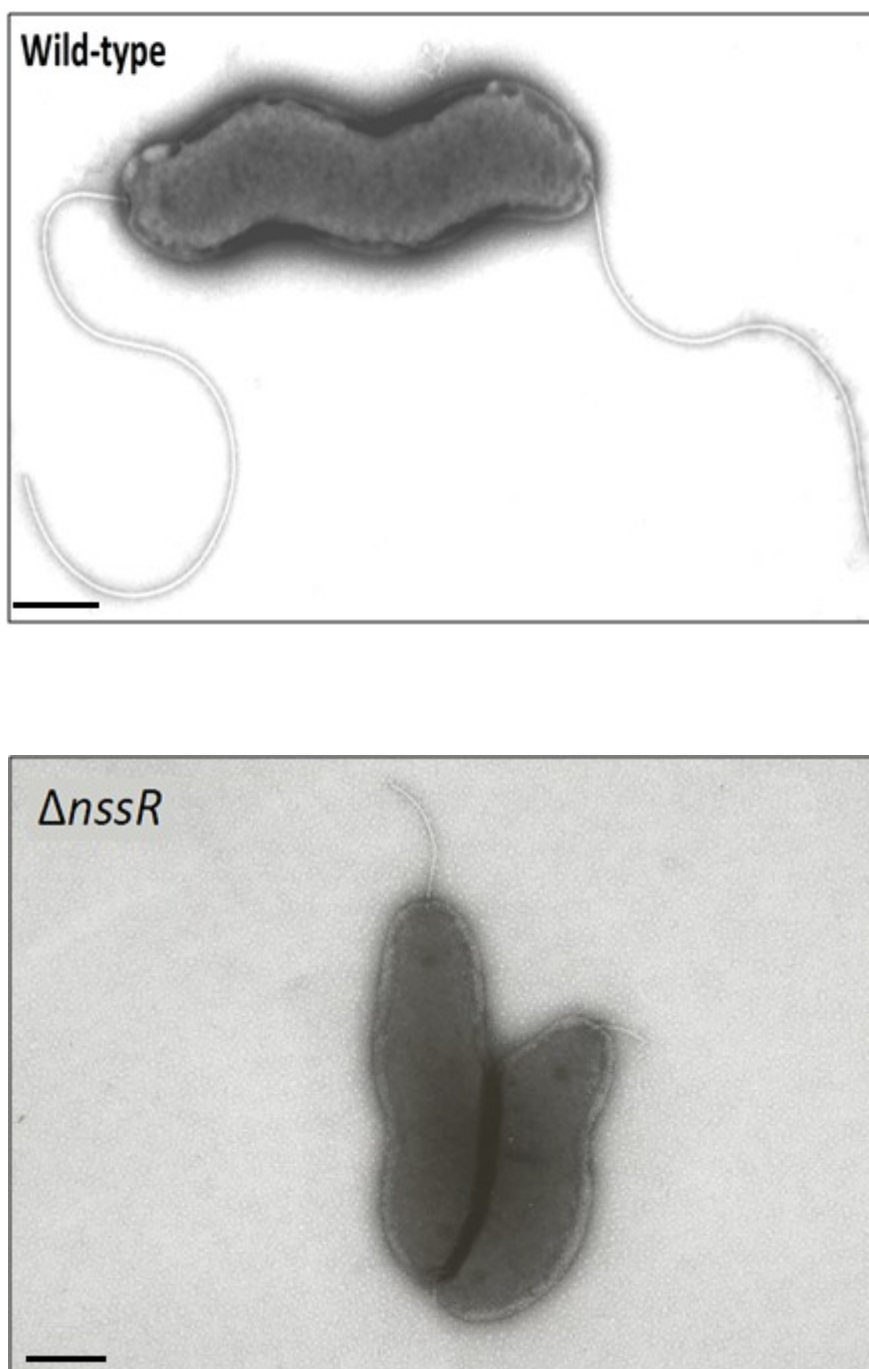
**Table 4.1 Mean diameters of zones of growth of 11168-O WT, 11168-O 0466<sup>-</sup> and 11168-O 0466C.** Strains were grown on 0.3% agar (Mueller-Hinton), for 48 hours.

Time (h)	Diameter of growth (mean $\pm$ standard deviation)		
	11168-O WT	11168-O 0466 <sup>-</sup>	11168-O 0446C
<b>24 (n= 14)</b>	42.57 $\pm$ 2.98mm	20.36 $\pm$ 3.34mm	46.57 $\pm$ 3.82mm
<b>36 (n= 10)</b>	51.00 $\pm$ 5.19mm	45.40 $\pm$ 4.01mm	53.60 $\pm$ 3.03mm
<b>48 (n= 9)</b>	75.56 $\pm$ 2.60mm	74.00 $\pm$ 2.24mm	77.00 $\pm$ 1.00mm

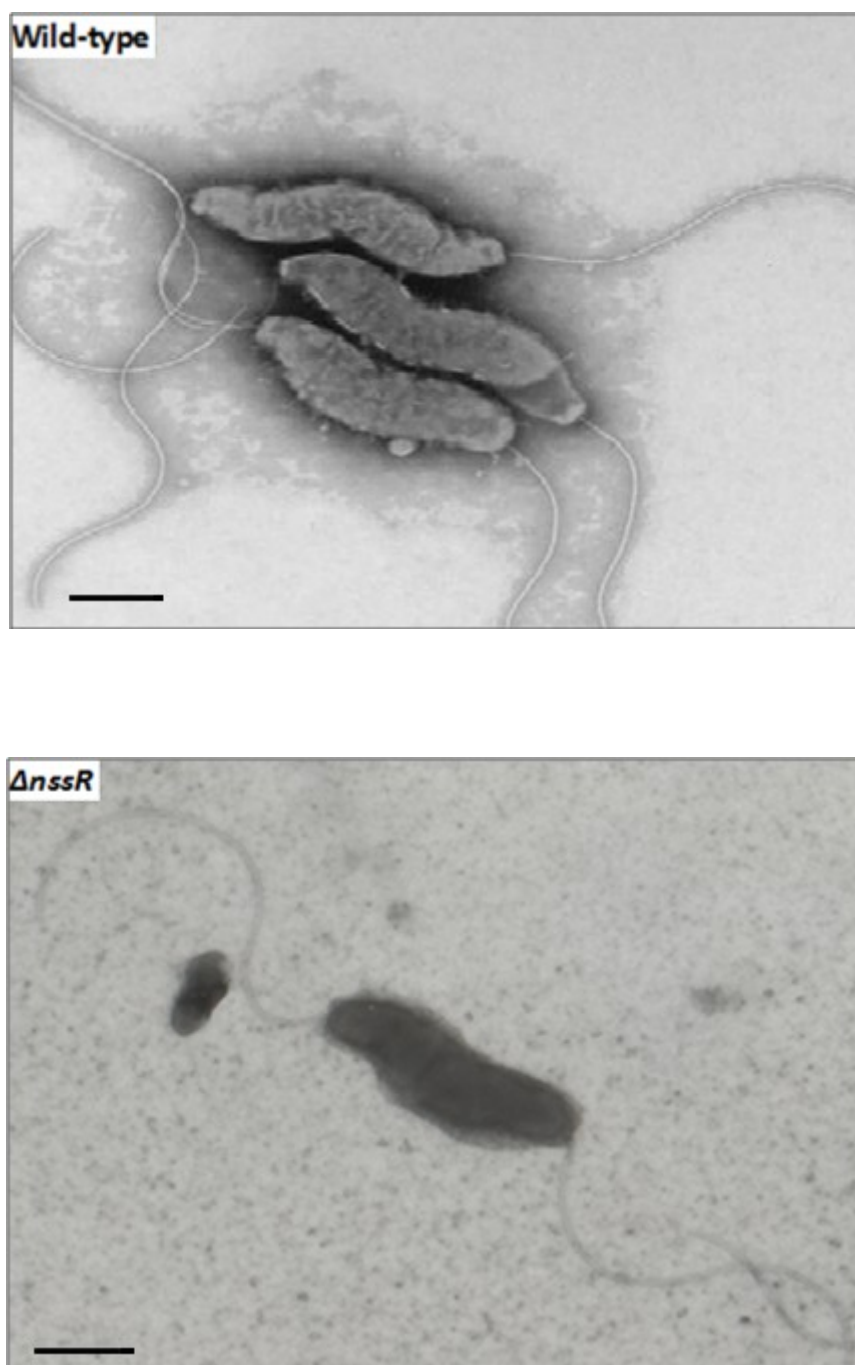


**Figure 4.1** Defect in 11168-O 0466<sup>-</sup> motility compared to the wild-type shown on motility agar. Growth of 11168-O WT, 11168-O 0466<sup>-</sup> and 11168-O 0466C after 24, 36 and 48h incubations on 0.3% MH agar plates.





**Figure 4.2. Wild-type and short asymmetrical flagella of 11168-O WT and 11168-O 0466<sup>-</sup> strains.** Electron micrographs of the 11168-O wild-type and  $\Delta nssR$  mutant taken from the outer edge of bacterial growth on 0.3% MH agar plates after a 24h incubation. Bars represent 200 nm.



**Figure 4.3. Wild-type flagella of 11168-O WT and 11168-O 0466<sup>-</sup>.** Electron micrographs of the 11168-O wild-type and  $\Delta nssR$  mutant taken from the outer edge of bacterial growth on 0.3% MH agar plates after a 48h incubation. Bars represent 400nm.

### 4.3.2 Video based measurement of motility in *C. jejuni* 11168-O background strains

#### 4.3.2.1 BacTracker

The motility of all strains of the *C. jejuni* 11168-O background was examined in a liquid culture using the BacTracker image processing software to calculate the mean velocity of the motile culture (Karim *et al.*, 1998). The ability of the software to track the movement of bacteria was found to be significantly impaired by even a small amount of drift (the current like movement of the growth medium across the slide), which made non-motile cells appear motile to the software and influenced the direction and speed of movement of motile cells, biasing the result. This was difficult to avoid when using conventional microscope slides and cover-slips as although leaving the slides for several minutes to allow the flow of liquid across the slide to stabilise decreased the amount of drift visible, it was not desirable to do this as *Campylobacter* cells quickly become less motile when exposed to atmospheric levels of oxygen and lower than optimal temperatures. Also, the measurement of velocity, which was calculated in this instance as an average value from all cells visible over the length of footage screened, was affected by the presence of non-motile cells in all of the cultures filmed. These cells may have been truly non-motile or simply stuck to the glass of the microscope slide or the cover slip, but all types of non-motile cells introduce error into the calculation of velocity, falsely lowering the average velocity of the culture. Microslides were used in an attempt to correct the drift, however this introduced an additional problem by increasing the depth of field visible on the video and so increasing the amount of non-motile and out of focus cells visible to the software, introducing yet more error for which the software was unable to compensate.

Despite these difficulties the following results were obtained, in mid log phase 11168-O WT bacteria had an average overall velocity of  $26.68 \pm 3.11 \mu\text{m}.\text{S}^{-1}$ , 11168-O

0466<sup>-</sup> bacteria had an average velocity of  $13.04 \pm 1.44 \mu\text{m}.\text{S}^{-1}$  and showed an increase in the numbers of spinning and tumbling cells, with fewer fast cells and an overall decrease in velocity, compared to the wild-type. 11168-O 0466C, complemented *ΔnssR*, bacteria had an average velocity of  $37.99 \pm 3.46 \mu\text{m}.\text{S}^{-1}$ , showing a marked increase in velocity from the wild-type which agrees with previous ‘by eye’ assessment of the motility of this strain in the laboratory. Due to the issues associated with drift, it was not possible to use this technology to gather any other information about motility in these strains, for example curvilinear and straight line velocities or linear displacement when spinning, which can be calculated by the software,

#### 4.3.2.2 Image J

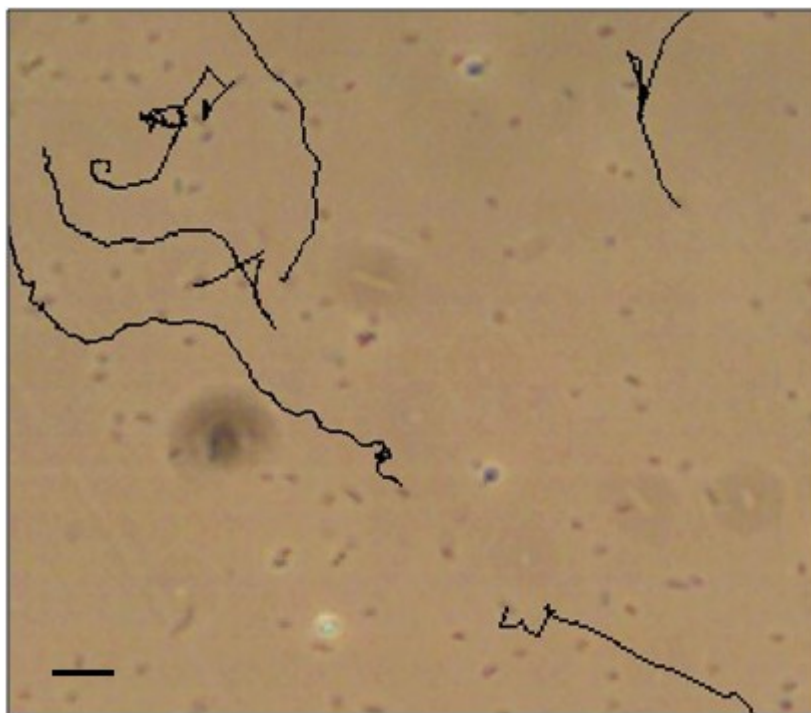
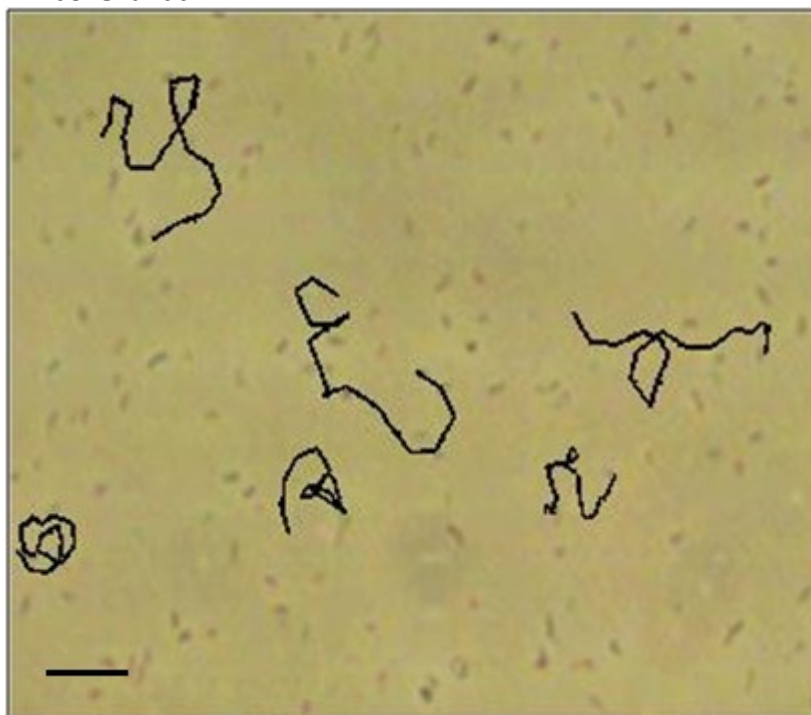
As an alternative to the BacTracker system the movement of individual 11168-O WT and 11168-O 0466<sup>-</sup> bacterial cells was recorded, traced and analysed using the ImageJ software with the manual tracking plug-in (Murray and Kazmierczak, 2006). The camera used for recording uses the Quick Time Motion JPEG format, which the Image J software is unable to process; therefore files were converted to .avi format using the 3GP converter software ([www.xilisoft.com](http://www.xilisoft.com)). These .avi files had then to be converted to the uncompressed .avi format using VirtualDub 1.7.0 ([www.virtualdub.org/](http://www.virtualdub.org/)) and finally were analysed with the ImageJ 1.37v software, using the manual tracking plug-in. The movement of six randomly selected cells, which remained in frame and in focus for the entire two seconds of video, were tracked for three separate films taken from three biological replicates.

As is evident from the representative tracking images shown in Figure 4.4, wild-type bacteria mainly showed the straight ‘darting’ swimming behaviour, with occasional tumbling, typical of *Campylobacter* and were more motile than the mutant cells, this

was seen in all of the clips tracked. 11168-O 0466<sup>-</sup> bacteria often appeared to spin around a fixed point and also to move in circular patterns with an increase in tumbling. Fewer cells in this strain appeared to move in straight lines compared to the wild-type and did not travel the same distance as wild-type cells, this was also observed in all of the footage of this strain tracked. The mean velocities calculated manually for each strain from the tracks of individual bacteria are given in Table 4.2; again the 11168-O 0466<sup>-</sup> mutant is significantly less motile than the 11168-O WT.

**Table 4.2. Mean and range of velocity of 11168-O WT and 0466<sup>-</sup> mutant cells as measured by manual tracking using the ImageJ software.** Calculated from tracks of log-phase broth cultures generated using ImageJ with the manual tracking plug-in (n=54).

Strain	Mean Velocity ( $\mu\text{m.S}^{-1}$ )	Lower range ( $\mu\text{m.S}^{-1}$ )	Upper range ( $\mu\text{m.S}^{-1}$ )
11168-O WT	$42 \pm 14$	33	61
11168-O 0466 <sup>-</sup>	$27 \pm 8$	15	38

**11168-O WT****11168-O 0466<sup>-</sup>**

**Figure 4.4. Defect in 11168-O 0446<sup>-</sup> motility compared to the 11168-O wild-type shown by video microscopy and tracking.** The movement of individual 11168-O WT and 11168-O 0466<sup>-</sup> cells was tracked for 60 frames (2s) in MH broth. Lines show the paths taken by a single cell during the 2s filming. Bar represent 5  $\mu$ m.

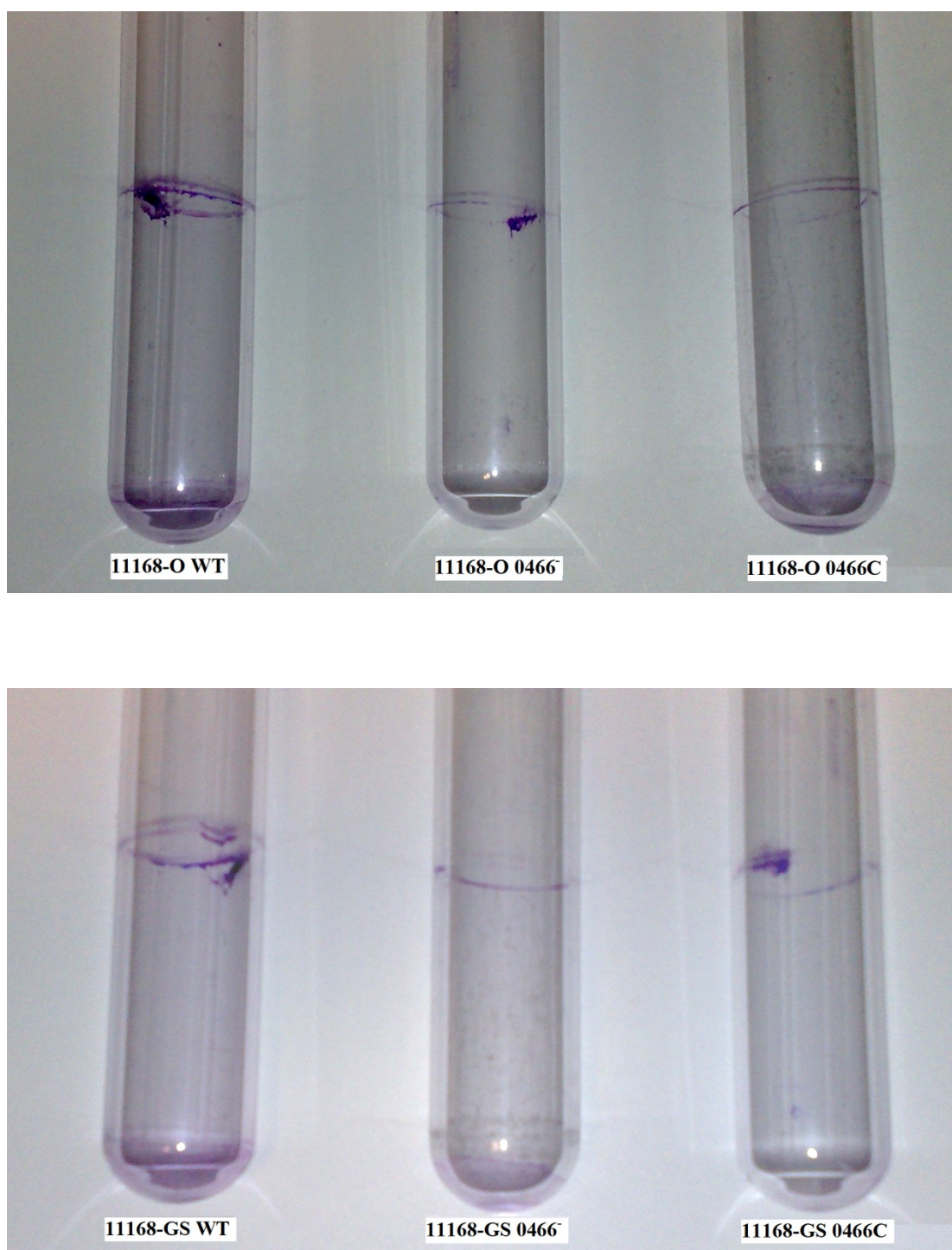
#### 4.3.3 Biofilm formation by *C. jejuni* 11168 strains is unaffected by *nssR* deletion.

The ability of all strains from the 11168-O and 11168-GS backgrounds to form biofilms or autoagglutinate was tested. Biofilm formation at the surface of a static broth culture produces a pellicle biofilm. Cells can also adhere to the glass surface of the test tube to form an attached biofilm or adhere to each other in the liquid media producing flocs, which can appear similar to cells that have autoagglutinated; whether these two processes are interlinked is unclear. The presence or absence of all types of biofilm was recorded daily, as was the number of replicates in which biofilms were formed.

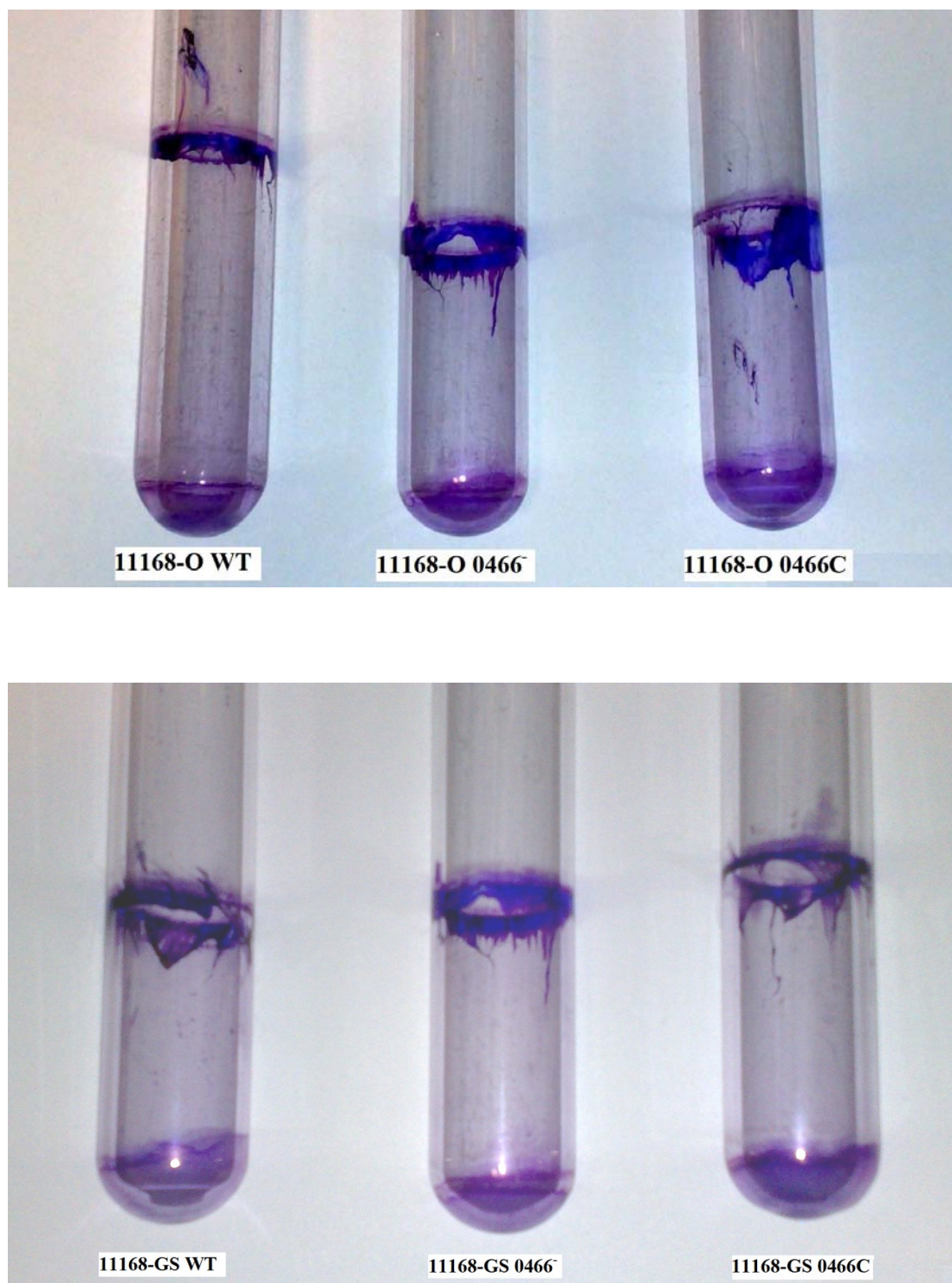
All strains repeatedly formed flocs after 72h incubation, pellicles and attached biofilms were formed after 96h in all cultures and this was the identical in all replicates. In all strains cells in the liquid phase of the culture became coccoid after 48h of incubation, whereas cells from the attached biofilms (prior to staining), pellicles and flocs, remained curved (11168-O background) or straight (11168-GS background) throughout the experiment. No evident difference in the size of the biofilms or the time taken for them to form was observed; no significant difference ( $P < 0.05$ ) was observed in the  $OD_{600nm}$  of the liquid phase of the cultures after any of the lengths of incubation tested. Representative images of the biofilms present after 4 and 6 day incubations are shown in Figures 4.5 and 4.6. It should be noted that during the development of this experiment various other conditions were tested, including the incubation of cells in the same manner as that of the final experiment but with shaking. In the liquid cultures with shaking at 100-120 r.p.m. no evidence of attached biofilm, pellicle, or aggregative floc formation was seen. The speed of shaking was decreased during testing until biofilm growth was first identified; in cultures which had been subjected to shaking at 40 r.p.m. no attached biofilms were observed, but flocs did form readily after incubation for 72h in the body of the culture in all strains; this could be attributed to either

autoagglutination or biofilm formation. Attached biofilms and pellicles were only observed when no shaking had taken place during incubation.





**Figure 4.5. Biofilm formation in *C. jejuni* strains after 4 days of incubation, no defects in the ability of strain to form biofilms are evident.** Cultures were incubated without shaking, the culture removed and the tube washed prior to staining with crystal violet.



**Figure 4.6. Biofilm formation in *C. jejuni* strains after 6 days of incubation, no defects in the ability of strain to form biofilms are evident.** Cultures were incubated without shaking, the culture removed and the tube washed prior to staining with crystal violet.

## 4.4 Discussion

### 4.4.1 Evaluation of methods used to measure motility.

#### 4.4.1.1 Motility agar

The results generated from the growth of strains on motility agar were most consistent when the inoculum was introduced directly into the medium, rather than dropped onto the surface of the agar and allowed to soak in; contamination was also reduced when using this variation. This method allows the easy identification of differences in the level of motility between strains, can provide quantifiable data, in the form of growth diameters, and also allows data to be presented visually. The major advantage of this technique is that further work can be carried out on a particular subsection of cells taken from the plate, i.e. from a hyper-motile out growth or from log-phase growth occurring at the edge of the zone of growth.

Major disadvantages of use of motility agar are that the characteristics of movement of individual cells cannot be assessed as it is an average property of the population that results in colony spreading. Furthermore the culture is not homogeneous and the activity of a particular subset of the population can not be examined. Also the motility of exponentially growing homogeneous cultures in liquid cannot be exactly modelled or assessed by the use of plates, as the change in medium unavoidably alters growth rate and introduces changes in environment, although the environment at the edge of a culture on a motility agar plate is thought to resemble that in a broth culture, at least to some degree. It is not possible to identify any differences in the type of motility, i.e. increased spinning or tumbling of cells. For these reasons the methods described below were also used to determine the motility behaviour of cells in homogeneous conditions and ideally, that of individual cells.

#### 4.4.1.2 The BacTracker

This system has the potential to be very advantageous to those wishing to measure bacterial motility as it is possible to greatly reduce the amount of manual calculation needed, however, technical problems can mean that it is difficult to obtain meaningful results. The BacTracker software has been used by Karim *et al.* (1998) to measure and compare the motility of *H. pylori*, *C. jejuni* and *E. coli*. It is possible to automatically and simultaneously track 120 moving bacteria and calculate data such as direction of movement, curvilinear velocity (length travelled by a bacterium divided by time taken), straight line velocity and curvature rate; the software can also compensate for Brownian motion.

In order to obtain reliable velocity data, it was necessary to obtain at least two, and preferably five, minutes of footage free of drift, which would alter the motion of the cells in a manner unconnected to their motility and so introduce error into the results. It proved impossible to obtain this length of footage using conventional microscope slides and cover-slips, as once the movement of liquid across the slide had ceased, the motility of the cells had decreased, possible due to too lengthy exposure to atmospheric concentrations of oxygen or room temperature.

Microslides, thin-walled capillary tubes with a rectangular profile and an internal width of 100µm which can be viewed under a microscope in the same way as a conventional slide, were used and successfully countered the problem of 'drift' but also introduced another technical issue. The 20µm depth of the tube greatly increased the depth of sample present compared to that of a conventional microscope slide, hence the depth of view visible on the video taken from these slides was also increased; this caused the majority of motile cells to move out of focus or out of frame before the software was able to calculate their velocity. The number of non-motile cells present

also affected the results, as in this case bacterial velocity was calculated as an average result for all of the cells present in the field of view, and due to the increased depth of view when using Microslides, more non-motile cells were visible on the video footage.

Whilst the BacTracker system has previously been used to calculate the velocities of several bacterial species (Karim *et al.*, 1998) the difficulties in obtaining representative video footage of motile cultures suitable for analysis by this software and the inability of the software to compensate for any imperfections in the footage, mean that this system is not suitable for use in this study, although if it were possible to overcome the technical difficulties this system would be ideal.

A system that could be used to analyse a shorter length of footage, so reducing the difficulties associated with obtaining the necessary amount of drift free footage and remove any decreases in motility associated with lengthy exposure to atmospheric concentrations of oxygen, would be preferable. It would also be advantageous for the user to be able to select manually which cells are tracked, a function which is performed automatically by the BacTracker software, so avoiding the loss of data that occurs when tracked cells move out of the field of view or out of focus and remove the error introduced by non-motile cells.

#### 4.4.1.3 Manual tracking using ImageJ

The ImageJ software with the manual tracking plug-in has been used by Murray and Kazmierczak (2006) to measure the motility of *AflhF* mutants in *P. aeruginosa*. This method generates traces of the movements of each tracked cell, which can be overlaid onto the original footage, which can be used to manually make calculations of the velocity of individual cells or to display the data visually, to show differences in motility that are not conveyed by velocity measurements alone (i.e. circular vs. linear

motion). Although all of the tracking of individual bacteria, and the majority of the calculations, have to be done by hand and thus can be quite laborious, the method is more robust and able to deal with problems as it allows more user input. The user is able to decide which cells to track, so removing any error introduced into the data by cells which leave the field of view or move out of focus. It is also possible to entirely eliminate any error caused by the presence of non-motile cells by simply not tracking these cells and as the lengths of footage used are very short, it is easy to avoid the problems generated by drift, as any parts of the footage affected can simply be avoided. To avoid introducing bias into the results it is necessary first to identify all of the cells suitable for tracking, such as those that remain in frame and in focus for the duration of the clip, then either track all cells or select a proportion at random.

Overall, manual tracking using the ImageJ system was found to be more suitable as a method of calculating bacterial velocity and visualising bacterial motility than the BacTracker system, for this application. Despite the lengthy manual calculations needed to obtain data, this data was less subject to error than that obtained from the BacTracker system and so more useful.

#### 4.4.2 Motility

Analysis of motility by all of the methods used here demonstrated that the 11168-GS background strains are not motile; this is consistent with the findings of Gaynor *et al.* (2004), who showed that the genome sequenced strain is non-motile, although this strain does retain full-length flagella. It was impossible to determine, from these experiments, whether there are any differences in motility that may indicate differences in flagellin glycosylation between the strains examined.



The work carried out here demonstrated that the 11168-O 0466<sup>-</sup> mutant has a significant defect in motility compared to the 11168-O WT, at least during exponential growth; this was evident in all of the methods used. In addition to being less motile the strain also moved in a different manner, with more circular movements and fewer long, fast runs than the wild-type. When the 11168-O 0466<sup>-</sup> mutant was grown on motility agar, electron microscopy of cells taken from the edge of the growth on the plate after 24h incubation appeared to show the growth of short, asymmetric flagella. Whilst neither flagellum had reached wild-type length, many cells had shorter flagella at one cell pole. Overall, spreading of the 11168-O 0466<sup>-</sup> mutant across the soft agar at 24h was significantly retarded, probably due to these short flagella. The difference was still significant at 36h on soft agar, although by 48h both strains had grown enough to cover almost the entire surface of an agar plate. It is likely that the shorter flagella are present at the younger pole of the cell, which is formed after cell division. As bacteria at the edge of the growth on soft agar plates would be dividing rapidly it is possible that the 11168-O 0466<sup>-</sup> cells do not have sufficient time for the synthesis of a full length flagellum to occur before cell division occurs again, leading to an asymmetry in flagellar lengths. This potentially points to a deficiency in flagellar biosynthesis in this strain.

Flagellin glycosylation is thought to have a role in flagellar formation, although this role has not yet been defined (Thibault *et al.*, 2001; Guerry *et al.*, 2006). As the 11168-O 0466<sup>-</sup> strain is believed to have alterations in flagellin glycosylation, it is possible that these alterations are affecting flagellar biosynthesis. The flagellar asymmetry may also be suggestive of a rate limitation in flagellar biogenesis which only becomes noticeable in rapidly growing cultures of the mutant strain. Cell division required the biosynthesis of a flagellum at the newly formed pole of the cell. Cells

growing at an increased rate, such as cells in the log-phase of growth, divide rapidly necessitating a fast rate of flagellar biosynthesis, if wild-type length flagella are to be produced. As such, any defect in the flagellar glycosylation machinery that decreases the speed of flagellar biosynthesis could lead to the presence of asymmetric flagella. The flagellum at the newer cell pole would be shorter in length than that at the older pole, as there would have been insufficient time between cell divisions for a wild-type length filament to form. When the rate of cell division slows the time between cell divisions increases, allowing longer flagellar filaments to be produced before the next division occurs. Once the rate of cell division decreases to the point where wild-type length filaments can be produced between cell divisions, or the rate limiting factor in flagellar biosynthesis is removed, the asymmetry in flagella disappears. The observation that full length flagella filaments were seen on 11168-O 0466<sup>-</sup> cells after 48h incubation would fit this hypothesis as by 48h, when the bacterial growth had reached the edges of the plates, the rate of cell division would have slowed considerably from that occurring at the edge of the zone of growth after 24h incubation. This decrease in growth rate would then allow the cell sufficient time to synthesise a full length flagellar filament at each pole before cell division occurred.

Whilst this hypothesis appears to fit the available data well, confirmatory evidence is difficult to obtain. It would first be necessary to define the mechanism of flagellin glycosylation in this strain, before identifying any defects present and determining whether these could be the limiting factor in flagellin glycosylation; this approach is beyond the range of this study. The changes in motility in the 11168-O 0466<sup>-</sup> mutant would appear to be due to the lack of expression of the FMR genes due to the deletion of the NssR transcriptional regulator. Although it is not possible to state this definitively, which would require genome wide NssR binding data from the ChIP-chip



work; it is not possible to say with any certainty whether this mutation has any other effects. The effects of mutation of the individual genes of the FMR alone in *C. jejuni* 11168 are largely unknown. In the closely related strain *C. jejuni* 81-176, mutants in *pseD* and Cj1334 were made by Karlyshev *et al.* (2002) and both mutants were fully motile. *pseD* is involved in the attachment of acetamidino pseudaminic acid to flagellin (Guerry *et al.*, 2006) and the function of Cj1334 is unknown.

Whilst it is clear that the 11168-O 0466<sup>-</sup> mutant is defective in motility in comparison to the wild-type, it is not possible to say exactly why. It is very likely that the lack of expression of the FMR genes, due to mutation of *nssR*, is responsible, but it is not possible to define which gene or genes active at which stage(s) of the flagellin glycosylation process are the cause of this defective motility.

#### 4.4.3 Biofilm formation

The aim of this part of the study was to establish whether there were any observable differences in the abilities of the strains used here to form biofilms or autoagglutinate. No differences in these abilities were noted, and all strains formed attached biofilms, pellicles and flocs in the body of the media in the same way, with the same level of efficiency and in the same amount of time.

The rationale for examining biofilm formation was that biofilms are often associated with stress tolerance and survival and as such may aid the survival of *Campylobacter* in both the environment and possibly in the host. Howard *et al.* (2009) identified a livestock clade associated glycosylation island (Cj1321-Cj1325/6) that was important in the colonisation of chicken and when mutated decreased the ability of the mutant to form biofilms and autoagglutinate. As campylobacters are microaerophilic, sensitive to desiccation and, in comparison to other foodborne pathogens, relatively

fragile, growth as a biofilm may be one of the mechanisms used by this bacterium for survival in otherwise lethal environments. The ability of *Campylobacter* to form biofilms *in vitro* has already been established (Trachoo *et al.*, 2002; Reeser *et al.*, 2007 and Joshua *et al.*, 2006), whilst monospecies biofilm formation by *Campylobacter in vivo* has not been observed, the presence of *Campylobacter* as components of multi-species biofilm is established (Trachoo *et al.*, 2002 and Keevil, 2003). Both flagella and quorum sensing components were found by Reeser *et al.* (2007) to be necessary for successful biofilm formation. Joshua *et al.* (2006) and Kalmokoff *et al.* (2006) also identified the flagellar filament as necessary for biofilm formation. Flagellin glycosylation has not been directly implicated in biofilm formation, other than that certain mutants that lack flagellin glycosylation and so are unable to synthesise a flagellar filament are unable to form biofilms. As the strains examined here show differences in flagellin glycosylation associated gene expression, but do not show any differences in biofilm formation, this work would appear to support the conclusion that flagellin glycosylation is not directly involved in biofilm formation. The finding that biofilms were not formed in the presence of shear forces, i.e. shaking, was also in agreement with the findings of Joshua *et al.* (2006).

Autoagglutination occurs spontaneously between *Campylobacter* cells and may be a preliminary stage of micro-colony and biofilm formation. Autoagglutination also acts as a marker of virulence in several Gram negative bacterial pathogens, including *Vibrio cholerae* (Chiang *et al.*, 1995) and *Bordetella pertussis* (Menozzi *et al.*, 1994). Pilins and outer membrane proteins associated with virulence in these species have also been shown to be autoagglutinins (Skurnik *et al.*, 1984). In *C. jejuni* 81-176 autoagglutination has been associated with flagella (Guerry *et al.*, 2002) and specifically with flagellar glycans (Guerry *et al.*, 2006), as a mutant in *pseD*, which lacked

acetamidino pseudaminic acid flagellar glycans, did not autoagglutinate; this was also observed in *C. coli* VC 167. The transposon mutagenesis carried out in *C. jejuni* 480 by Golden and Acheson (2002) also identified *pseD* and Cj1318 mutants as unable to autoagglutinate. The glycosylation island (Cj1321-Cj1325/6) identified by Howard *et al.* (2009), in and particularly *ptmG* (Cj1324), one of the genes that make up the island, has a role in autoagglutination, biofilm formation and the colonisation of chickens. Given that the 11168-O 0466<sup>-</sup> mutant shows no expression of either *pseD*, Cj1318 or the genes of the glycosylation island, it was hypothesised that these experiments would find a defect in autoagglutination in this strain, which would be consistent with previous work in other *Campylobacter* strains. Although the method used here could not distinguish between autoagglutination and biofilm formation in the body of the culture medium, any differences in the types of growth seen would have led to further work to determine whether this was due to biofilm growth or autoagglutination. As no differences in the growth of this or any other strain tested were seen, it can be concluded that either the method is unsuitable for this type of analysis, or that the hypothesis that the lack of expression of *pseD*, Cj1318 and Cj1321-Cj1325/6 would alter autoagglutination in this strain is incorrect. As the method used is similar in principle to that used by Guerry *et al.* (2002) and Joshua *et al.*, (2006) to assess autoagglutination and biofilm formation respectively, it is reasonable to expect that this method would be able to detect any gross differences in biofilm formation or autoagglutination present. Another possible explanation is that, as the flagellin glycosylation locus in *C. jejuni* 11168 is the largest and most complex yet identified in *Campylobacter*, the mutations which lead to loss of autoagglutination in other strains of *Campylobacter* are compensated for by differences in glycosylation in this strain. The *C. jejuni* 11168H strain in which the glycosylation island (Cj1321-Cj1325/6) was characterised by

Howard *et al.* (2009) is a hyper-motile derivative of the 11168-GS WT strain which is thought to be closely related to the 11168-O WT strain. Differences in flagellin mass and antigenicity, probably due to altered glycosylation profiles have been observed between the strains (Turner and Penn, 2005). It is possible that these differences may also account for the differences observed in AGG and biofilm formation.

# Chapter V: Analysis of *C. jejuni*

## 11168 flagellar glycans

### 5.1 Introduction

A recent study by Logan *et al.* (2009) identified a series of glycosyl residues present on *C. jejuni* 11168 flagellin, which include the pseudaminic and legionaminic acid derivatives PseAc, PseAm, LegAm and MeLegAm which have previously been identified decorating *C. jejuni* 81-176 and *C. coli* VC67 flagellin. In addition to these previously recognised modifications two novel glycans, both di-*O*-methyl-glyceric acid derivatives of pseudaminic acid and its derivative acetamidino pseudaminic acid, were also discovered; these novel glycans were found to be the most common modifications present on *C. jejuni* 11168 flagellin. For more details of these glycans see Table 1.1 (Section 1.8). Whilst the different glycans found on *C. jejuni* 11168 flagellin were identified, no ‘map’, detailing the locations of the flagellar glycans on the amino acid backbone of the flagellin protein, has been published. Another interesting analysis that has yet to be undertaken would be a comparison of the flagellar glycans present in the two backgrounds of *C. jejuni* 11168, the ‘genome sequenced’ 11168-GS and ‘original’ 11168-O strains. The flagellin glycosylation region of *C. jejuni* 11168 is the largest yet discovered in *Campylobacter*, whilst some genes thought to be associated with flagellin glycosylation have been assigned functions based upon their homology to genes characterised in other *Campylobacter* strains, there remain many genes which have no clear function, despite the evidence linking some to flagellar glycosylation or motility.

Hence there is still much regarding flagellar glycosylation in *C. jejuni* 11168 that remains unclear.

Recent work by Howard *et al.* (2009) identified a glycosylation island (Cj1321 to Cj1325/6) which was found to be important for several cellular processes including the biosynthesis of certain flagellar glycans and in the successful colonisation of chickens. In particular a  $\Delta$ Cj1324 mutant in the 11168H strain background, a hypermotile variant of the sequenced strain 11168-GS WT (Karlyshev *et al.*, 2002), was found to lack a 315Da flagellar glycan which, because of their identical masses, could correspond to either PseAm or LegAm. Cj1324 has previously been found to have a role in legionaminic acid biosynthesis in *C. coli* VC167 and hence has been annotated *ptmG*; therefore it is likely that the glycan lost is the legionaminic acid derivative, LegAm. The loss of this glycan from 11168H flagellin caused a decrease in the ability of cells to autoagglutinate and a decrease in hydrophobicity, probably due to a change in cell surface charge caused by the changes in the flagellum; the ability of the strain to colonise chickens was also significantly reduced. The mutation of Cj1321 and Cj1325/6 in this strain had no observable effect on autoagglutination or hydrophobicity and the functions of these genes, together with Cj1322 and Cj1323 the other genes of the glycosylation island, has yet to be deduced. Previous work in this thesis, detailed in Chapter 3, found that whilst Cj1321, Cj1324 (*ptmG*) and Cj1325/6 were transcribed; there was no evidence of expression of Cj1322 or Cj1323 in 11168-O WT. This study also identified several of the same flagellar glycans as the work by Logan *et al.* (2009) which used metabolomics analysis and mass spectrometry of flagellin. Work by Guerry *et al.* (2006) also used MS/MS to characterise a Cj1333 mutant in *C. jejuni* 81-176 and found that this mutant had lost the ability to decorate flagellin with PseAm. This work led to the conclusion that Cj1333 is somehow involved in the attachment of PseAm to

flagellin and hence Cj1333 was re-annotated as *pseD*, part of the *pse* family of pseudaminic acid associated genes.

The FMR, the region of 17 genes, from Cj1318 to Cj1336, down-regulated in the *nssR* mutant in the original strain background of *C. jejuni* 11168, contains several genes known to be involved in flagellin glycosylation, including the *ptmA-H* genes, involved in legionaminic acid synthesis in *C. coli* VC167, and *pseD* which is implicated in the transfer of acetamidino pseudaminic acid to flagellin in *C. jejuni* 81-176; also present are other genes that are still to be characterised. Given the studies detailed before and work by others, it is possible to begin to predict the effects that the loss of expression of the FMR in the 11168-O 0466<sup>-</sup> mutant should have on the flagellar glycans. However, as well as the potential loss of PseAm and the legionaminic acid derivative LegAm there may also be other glycosylation differences between this and the wild type strain, as many of the down-regulated genes remain uncharacterised. As such, one aim of this part of the study is to begin to map the glycans present on 11168-O WT and 11168-O 0466<sup>-</sup> flagellin by mass spectrometry. Any differences observed in the number, type or location of flagellar glycans may help to explain the defective motility and altered flagellar structure of the 11168-O 0466<sup>-</sup> mutant, as well as the decrease observed in 11168-O 0466<sup>-</sup> flagellin mass in comparison to the wild-type when analysed by SDS-PAGE (Turner and Penn, 2005). The examination of 11168-O WT flagellin will also allow the mass spectrometry method, which has not previously been used for the analysis of flagellin glycopeptides, to be evaluated. The 11168-GS WT will also be examined, with the aim of identifying any differences in glycosylation between the wild-types of the two *C. jejuni* 11168 backgrounds.

As detailed in Section 4.3.1, some 11168-O 0466<sup>-</sup> mutant cells growing at the edge of a soft ‘motility’ agar plate developed short, asymmetric flagella after

approximately 24 hours of incubation, however, after incubation for 48 hours, the flagella of these cells reached wild-type length and no asymmetry was visible. A possible explanation of these observations is that the asymmetry of the flagella is due to a defect in flagellar glycosylation in this strain which decreases the rate of flagellar biosynthesis, and is made noticeable only because cells taken from the edge of the zone of growth of a soft agar plate are growing at a fast rate, possibly exponentially; this is discussed further in Section 4.4.2. A comparison of the type, number and location of the various glycans present on the flagellin of the 11168-O 0466<sup>-</sup> mutant taken from cells in the exponential and stationary phases of growth should show whether this potential defect in flagellar biosynthesis is due to changes in glycosylation or to other unknown mechanisms. Therefore another aim of this part of the project is to begin to map the glycans present on 11168-O 0446<sup>-</sup> flagellin isolated from exponential and stationary phase cells.

To adequately describe glycosylation, three pieces of information are required. Firstly the identification of the glycosylated proteins and peptides, secondly the site(s) of the glycosylations on the peptides and thirdly the structure of the glycan(s). The use of mass spectrometry to identify glycosylation sites has been previously described (Guerry *et al.*, 2006; Thibault *et al.*, 2001 and others) but can be very challenging, as the glycosyl modifications present can be heterogeneous and do not give the clearly defined mass shifts characteristic of other types of post-translational modifications, for example phosphorylation and acetylation; obtaining structural information from mass spectrometry analyses is also difficult.

Many studies which aim to identify flagellar glycans use a targeted metabolomics approach together with mass spectrometry to deduce the structure and location of flagellar glycans. This approach involves first extracting intracellular sugar-



nucleotides from the strain to be examined, then probing these by mass spectrometry for those nucleotide-activated metabolites which are relevant to the biosynthesis of pseudaminic and legionaminic acids and their derivatives. Any metabolites of potential interest are then purified and analysed by nuclear magnetic resonance (NMR) spectrometry to determine their identity. Mass spectrometry is then used again to confirm the presence of these glycans on flagellin by the analysis of flagellin glycopeptides. The resulting mass spectra are searched for glycan residues, located on serine or threonine residues only, of identical mass to those identified by the NMR spectroscopy. Studies of this type have been carried out on the flagellin of *C. coli* VC167 (McNally *et al.*, 2007), *C. jejuni* 81-176 (McNally *et al.*, 2006a), *H. pylori* (Schirm *et al.*, 2003) and most recently *C. jejuni* 11168 (Logan *et al.*, 2009 and Howard *et al.*, 2009). Whilst a complete targeted metabolomics approach is beyond the range of this study, it is also unnecessary, as many glycans isolated from *Campylobacter* flagellin have already been identified by those studies detailed above, including those of *C. jejuni* 11168 (Logan *et al.*, 2009). Hence, glycopeptides of *C. jejuni* 11168 flagellin will be examined for the presence of known flagellar glycans in a method similar to that used in the later stages of the above detailed metabolomics method. Collision-induced dissociation (CID) and electron-capture dissociation (ECD) mass spectrometry in particular will be used to locate flagellar glycans.

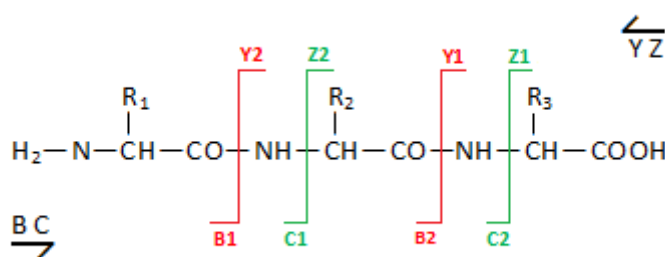
Briefly, mass spectrometry involves firstly the digestion of the protein to be analysed with the protease trypsin, which cleaves protein at the carboxy termini of both arginine and lysine amino acid residues. As such, all cleaved peptides contain either a basic arginine or lysine residue and are therefore suitable for the positive ionisation which forms the next part of the process. Following this, the ions of the sample peptides are separated according to their mass (*m*) and charge (*z*) ratio, this separation is

recorded as a mass-charge ( $m/z$ ) spectrum. For simple MS analysis this  $m/z$  spectrum can be used as a 'protein fingerprint' to identify the sample, as two different proteins are unlikely to fragment in the same way, hence a  $m/z$  spectrum is characteristic of a particular protein. In more complex tandem mass spectrometry (MS/MS), such as that carried out here, the individual groups of particular peptide species, which have been separated according to their mass and charge, are fragmented within the mass spectrometer. These fragment ions are separated according to their mass ( $m$ ) and charge ( $z$ ) ratios and this separation is again recorded as a mass-charge ( $m/z$ ) spectrum. This spectrum of the resulting fragment ions can be used to generate structural information regarding the original, intact, sample molecule. For example, the sequence of amino acid residues within a protein can be determined by analysing the fragment ions generated from the fragmentation of its component peptides.

Two different MS/MS methods are used in this study to attempt to locate glycan residues, the first method is collision-induced dissociation (CID) MS/MS, which is a technique used extensively for protein identification and is able, generally, to give good coverage of the peptides within a sample, to produce a relatively complete amino acid sequence. However, this technique preferentially cleaves glycan residues rather than the peptide backbone when analysis of glycopeptides is carried out, leaving the peptide backbone largely intact (Huddleston *et al.*, 1993). As such, this technique is suitable for either the characterisation of unglycosylated peptides or glycans separately, but not the analysis of glycopeptides. It is not likely to be capable of determining which amino acid residues are glycosylated as it is unlikely to show the loss of a complete glycan from a peptide, together with the fragmentation of that peptide, which is necessary to identify the glycosylated residue (Alley *et al.*, 2009). Due to the limitations of CID, another method, electron-capture dissociation (ECD) MS/MS, will also be employed. ECD

induces extensive fragmentation of the peptide backbone but only limited fragmentation of the glycan residues, which are retained largely intact on the amino acid residue, allowing the site of the modification to be located (Zubarev, 2004). However, due to the extensive fragmentation of the peptide backbone it can be difficult to obtain complete peptide sequence data using this method.

Hence a mass spectrometry approach combining both CID, aiming to give good coverage of the amino acid sequence of the glycopeptides, with ECD, to provide the location and identification of any glycans present, will be used to attempt to map the locations and types of glycans present on *C. jejuni* 11168 flagellin, with the overall aim of linking differences in flagellin glycosylation between these strains to the motility, flagellin mass and gene expression differences observed.



**Figure 5.1. Fragmentation of a peptide precursor ion to generate, B', C', Y' or Z' ions.**

When peptide ions are subjected to CID mass spectrometry, which uses high pressure, energised helium gas to induce fragmentation, the OC-NH bonds of the peptide backbone are usually the first bonds to break, this generates B' and Y' ions, as indicated in red in Figure 5.1 above. If a singly charged peptide ion is fragmented the single charge can be retained on only one of the resulting fragments, if the charge is

retained on the N-terminal fragment it becomes a B' ion, if the charge is retained on the fragment C-terminal of the breakage then the ion is named a Y' ion. The B' and Y' ions are numbered based upon location of the bond which has been broken. The fragment which does not retain the charge from the precursor becomes invisible to the mass spectrometer as it can no longer be manipulated. A multiply charged ion can generate both B' and Y' ions, or a single, multiply charged, ion. When CID of glycopeptides is carried out the C-C bonds of the glycan may fragment before the peptide bonds, which can hinder the assignment of a modifying glycan to a particular residue.

ECD mass spectrometry uses a stream of electrons to induce fragmentation of the peptide backbone, as electrons are used all ions subjected to ECD must have multiple positive charges. In ECD the HN-CH bonds are usually broken preferentially to produce C' and/or Z' ions, indicated in Figure 5.1 above in green. ECD preferentially produces C' ions resulting from the charge being retained on the N-terminal fragment, the mechanism for this is unknown. When the charge is retained on the C-terminal fragment the resulting ion is termed the Z' ion, numbering is again based on the location of the broken bond, counting from either the N- or C- terminus depending on the type of ion and hence the terminus upon which the charge was retained after fragmentation. Again, multiply charged precursor ions can generate both C' and Z' ions.

## 5.2 Methods

### 5.2.1 *C. jejuni* flagella purification

#### 5.2.1.1 Growth conditions

*Campylobacter* strains 11168-GS WT, 11168-O WT and 11168-O 0466<sup>-</sup> were grown in MH broth or on MH agar at 37°C in a microaerobic environment as previously described. Over-night cultures were used to inoculate 400 ml MH broth to an OD<sub>600nm</sub> of 0.05; this culture was then grown until an OD<sub>600nm</sub> of approximately 0.3 was reached. The 11168-O 0466<sup>-</sup> strain was also grown on MH agar for 24 hours and colony material was then removed from the plates using a sterile loop and emulsified into 5 ml Tris-NaCl (10mM TrisHCl 0.85% (w/v) NaCl pH 7.4).

#### 5.2.1.2 Flagella filament isolation

This was adapted from the method of Power *et al.* (1994). 24h broth cultures were centrifuged at low-speed, 6 000 x g for 10 minutes at 4°C to pellet cells. The pellet was then resuspended in Tris-NaCl (10mM TrisHCl- 0.85% (w/v) NaCl, pH 7.4) to a calculated OD<sub>600nm</sub> of 20. The resulting suspension was homogenised for 3 minutes using an Ultra-Turrax T-25 high speed homogeniser (IKA Labortechnik) at a speed of 20,500 revolutions.min<sup>-1</sup>. Whole cells and cell debris were then removed by two low-speed centrifugations at 6 000 x g for 20 minutes at 4°C in a total volume of 20ml. The resulting supernatant was subjected to ultracentrifugation at 100 000 x g for 60 minutes at 5°C. The pellet was then resuspended in 1% (w/v) SDS in distilled water, incubated at 37°C with gentle agitation for 2h and again subjected to ultracentrifugation as above. The SDS washing was repeated three times, after which the flagella fragments were checked for purity by Coomassie blue-stained SDS-PAGE gels and electron

microscopy. The final pellet was then washed by resuspension and centrifugation three times in distilled water and stored in distilled water at -20°C until required.

## 5.2.2 Mass spectrometry

### 5.2.2.1 Excision of the flagellin bands

Flagellar proteins isolated from *C. jejuni* strains were separated by polyacrylamide gel electrophoresis. Bands with a molecular mass of approximately 60kDa were cut out of the gel with clean, sharp scalpel blades, using a separate blade for each band and taking care not to cross contaminate gel slices. The slices were collected in a 48-well plate and stored at -20°C until required for trypsinisation.

### 5.2.2.2 Trypsinisation

Modified trypsin is an enzyme used for the cleavage of proteins prior to analysis by mass spectrometry. It is used to generate peptides that are within the mass range of the spectrometer and which can be fragmented efficiently. Modified trypsin cleaves the peptide bonds in proteins on the c-terminal side of both lysine and arginine residues; the enzyme is modified to reduce non-tryptic activities and prevent autolysis. Trypsinisation was carried out in 48 well v-shaped plates using a MWGAG BIOTECH Robo Seq 4204 S liquid handling robot. The following reagents and protocols were used.

#### Reagents:

Digestion buffer	100mM ammonium bicarbonate
Destain solution	50% (v/v) acetonitrile in 0.5 x digestion buffer
Reduction buffer	10mM DTT in digestion buffer
Alkylation buffer	55mM iodoacetamide in digestion buffer
Trypsin solution	6 ng.µl <sup>-1</sup> modified trypsin (Promega, Madison, USA) in 0.5 x digestion buffer
Extraction buffer	1% (v/v) formic acid
	2% (v/v) acetonitrile in water

ProtocolsProgram one - Trypsinisation

Dehydration	Dispense 80 µl acetonitrile, wait 5 minutes, aspirate 160 µl.
Destain	Dispense 50 µl digestion buffer, dispense 50 µl acetonitrile, wait 10 minutes, aspirate 120 µl, wait 5 minutes.
Dehydration	Dispense 50 µl acetonitrile, 5 minutes, aspirate 70 µl, wait 5 minutes.
Reduction	Dispense 50 µl reduction buffer, wait 30 minutes.
Alkylation	Dispense 50 µl alkylation buffer, wait 20 minutes, dispense 100 µl acetonitrile, wait 5 minutes, aspirate 200 µl.
Wash	Dispense 50 µl digestion buffer, wait 10 minutes, dispense 50 µl acetonitrile, wait 15 minutes, aspirate 100 µl
Dehydration	Dispense 50 µl acetonitrile, wait 5 minutes, aspirate 70 µl, wait 5 minutes
Trypsin digestion	Dispense 25 µl trypsin solution

The lid of the 48-well plate was sealed with parafilm and the plate incubated at 37°C for 2.5h, 15 µl HPLC grade water was then added to each well and the plate incubated at 37°C overnight. The wells were checked periodically to ensure that the gel slices had not dried out. Following the incubation peptide extraction was carried out.

Program two - Peptide extraction

The processed gel slices from program 1 were returned to the robot in the 48-well plate, and the following program was then carried out:

Extraction one	Dispense 30 µl extraction buffer, wait 30 minutes
Peptide removal one	Aspirate 23 µl and dispense into final plate
Extraction two	Dispense 12 µl extraction buffer and 12 µl acetonitrile, wait 30 minutes.
Peptide removal two	Aspirate 23 µl from each well and dispense in final plate

### 5.2.3 Liquid Chromatography

Samples were injected using an online Micro AS auto-sampler and Surveyor MS pump (Thermo Fisher Scientific, Epsom, UK). Peptides were separated using a binary solvent system consisting of solvent A: comprising 0.1% formic acid (J.T. Baker, Holland) in water (J.T. Baker, Holland) and solvent B comprising acetonitrile (J.T. Baker, Holland) and 0.1% formic acid. A linear 40 min gradient, which increased the composition of (B) from 5% to 40%, was used together with an Integrafrit (New Objective, USA) C8 resolving column with a length of 10 cm and an internal diameter of 75  $\mu\text{m}$ . The peptide was introduced into the mass spectrometer using a Triversa Nanospray source (Advion Biosciences, NY) with an elution in a flow rate of  $\sim 300 \text{ nL min}^{-1}$ ; the typical nanoelectrospray voltage was +1.7 kV.

### 5.2.4 Mass spectrometry

A 7-T LTQ FT mass spectrometer (Thermo Fisher Scientific, Epsom, UK) was used to perform a data-dependent scanning. Data acquisition was controlled by the Xcalibur 2.0 software.

#### 5.2.4.1 Collision-induced dissociation (CID)

The mass spectrometer alternated between a full FT-MS scan ( $m/z$  380 – 2000) and subsequent CID MS/MS scans of the three most abundant ions. Survey scans were acquired in the ion cyclotron resonance (ICR) cell with a resolution of 100 000 at  $m/z$  400. Precursor ions were isolated and subjected to CID in the linear ion trap, the product ion was acquired with a resolution of 25 000 at  $m/z$  400. Automatic gain control (AGC) target values for full scan acquisition was maintained at  $1\text{e}^6$  counts for detection in the ICR cell and  $5\text{e}^5$  counts for detection in the linear ion trap. Collision activation for the



experiment was performed using helium gas at collision energy normalized to precursor  $m/z$  of 35% and  $q_{\text{excite}} = 0.25$ . The width of the precursor isolation window was  $m/z$  2 and only multiply charged precursor ions were selected for MS/MS. Dynamic exclusion, which prevents reanalysis of a precursor ion, was 180 s.

#### 5.2.4.2 Electron-capture dissociation (ECD)

The mass spectrometer alternated between a full FT-MS scan ( $m/z$  380 – 2000) and subsequent ECD MS/MS scans of the first most abundant ions. Survey scans were acquired in the ICR cell with a resolution of 100 000 at  $m/z$  400. Precursor ions were isolated in the ion trap and transferred to the ICR cell. Isolation width was 6  $m/z$  and only multiply charged precursor ions were selected for MS/MS. Dynamic exclusion used was 180 s. AGC target values for full scan and ECD acquisition was maintained at  $1e^6$  counts for detection in the ICR cell. In the ICR cell the ions were subjected to ECD, which the product ion was acquired with a resolution of 25 000 at  $m/z$  400. The electrons for ECD were produced by an indirectly heated barium tungsten cylindrical dispenser cathode (5.1 mm diameter, 154 mm from the cell, 1 mm off-axis). The current across the electrode was  $\sim 1.1$  A. Ions were irradiated for 60 ms at 5% energy (corresponding to a cathode potential of -2.775 V). Each ECD fragmentation was performed using 6 microscans.

#### **5.2.5 Data analysis**

CID and ECD MS/MS data were searched against the NCBI non-redundant database using the SEQUEST algorithm within Bioworks 3.3.1 (Thermo Electron Corp., Bremen, Germany). The search used the monoisotopic values 315.293 (acetamidino pseudaminic acid), 316.3394 (pseudaminic acid), 329.1164 (legionaminic

acid derivative, LegAm), 315.1324 (legionaminic acid derivative, MeLegAm), 390.1270 (dimethylglyceric acid derivative of PseAc) and finally 389.1274 (dimethylglyceric acid derivative of PseAm; Logan *et al.*, 2009) to attempt to identify potential glycan modifications of the serine and threonine amino acid residues only.

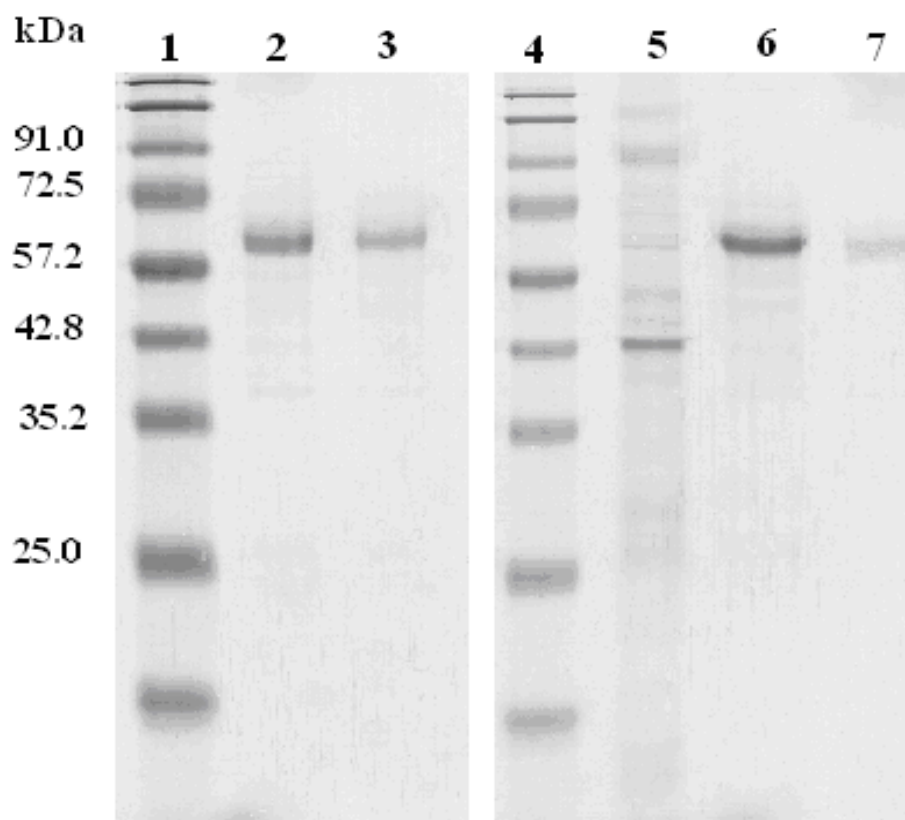
## 5.3 Results

### 5.3.1 Isolation of flagella filaments.

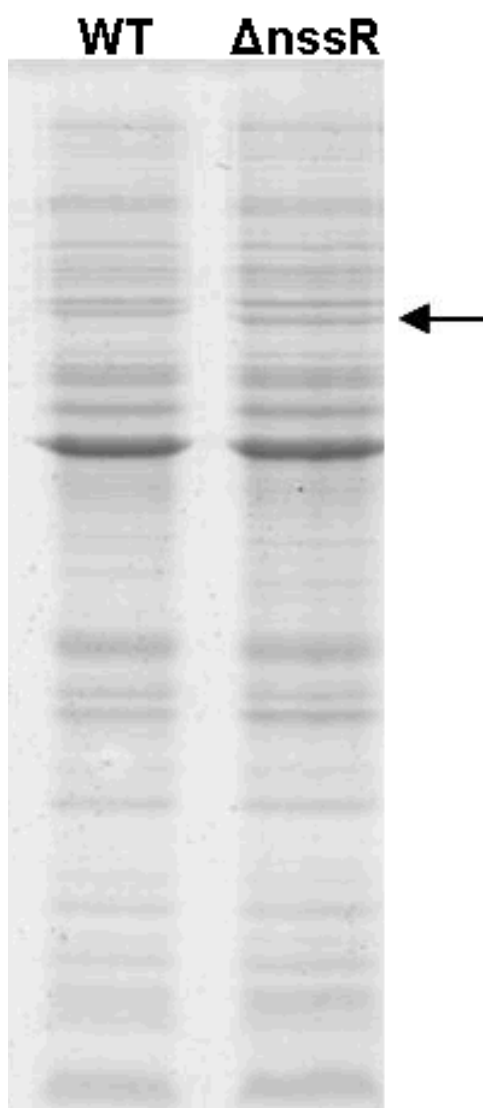
It was found to be impossible to isolate enough flagellin from 11168-O 0466<sup>-</sup> cells taken from the outside of the zone of growth on motility agar for the sample to be identified as flagellin when analysed by MS/MS. Therefore cells isolated from early log-phase broth cultures were used as an alternative, to provide flagellin for analysis from exponentially growing cells of this strain. Whilst this change of growth medium and conditions will affect the growth rate, and potentially the flagellar glycans present in this strain, it is the best approximation of soft agar growth conditions available. Flagellar filament pieces were also isolated from log-phase broth cultures of 11168-O WT, 11168-GS WT and plate grown cultures of 11168-O 0466<sup>-</sup>, to provide flagellin from stationary phase cells of this strain. All flagellar pieces were purified to homogeneity by an adaptation of the method given of Power *et al.* (1994).

Figure 5.2 shows an SDS-PAGE gel of the isolated, purified flagella fragments, which indicates that this method allows for the purification of flagellin to apparent homogeneity. The flagellin band in lane seven, corresponding to the flagellar pieces isolated from 11168-O 0466<sup>-</sup> mutant cells grown in broth, contains a relatively small amount of protein, this is potentially due to the presence of short flagella on 11168-O 0466<sup>-</sup> mutant cells. Another feature of this gel is the the breadth and diffusion of the flagellin bands themselves, which could potentially indicate the presence of a greater heterogeneity in flagellin mass from an individual strain than would perhaps be expected. As the flagellin proteins themselves should be of an identical mass, it is likely that there are differences in the number and/or nature of the glycosylation residues present. It is possible that this heterogeneity is due to either the loss of glycans during the purification process or to natural variability in the numbers or types of glycans

applied to the flagellin during the glycosylation process. If the later is the case, it is possible that the same variation could be visible in native material extracted directly from cells. To test this, 10 $\mu$ l of whole cell lysate of 11168-O WT and 11168-O 0466<sup>-</sup> mutant cells, taken from Muller-Hinton broth cultures with an OD<sub>600nm</sub> of 0.2, was analysed by SDS-PAGE, the resulting gel is shown in Figure 5.3. The decreased mass of the 11168-O 0466<sup>-</sup> flagellin band is evident, in comparison to its parent. As the FlaA and FlaB sequences of these strains are identical it is likely that this difference in mass is due to differences in glycosylation. However, whilst the amount of material applied to the gel was relatively small, the flagellin band, indicated by the arrow, is very sharp. If variation in flagellin glycosylation were occurring naturally within a strain it is likely that this band would be broader and more diffuse.



**Figure 5.2. SDS-PAGE analysis of isolated flagellar pieces.** 12.4% SDS-PAGE gel, stained with Coomassie blue, 4 $\mu$ l of purified flagellar pieces were applied to each lane. Lanes 1 & 4 –molecular weight markers,  $M_r$  values given. Lane 2- 11168-GS WT. Lane 3- 11168-O WT, Lane 5- whole cell protein extract from 11168-O WT, for comparison. Lane 6- 11168-O 0466<sup>-</sup> grown on agar. Lane 7- 11168-O 0466<sup>-</sup> grown in broth.



**Figure. 5.3.** SDS-PAGE gel showing the difference in mass between 11168-O WT and 11168-O 0466<sup>-</sup> mutant flagellin. Lane 1, 10μl 11168-O WT cell lysate. Lane 2, 10 μl 11168-O 0466<sup>-</sup> cell lysate. The arrow indicates the flagellin band.

### 5.3.2 Mass Spectrometry (MS)

When analysed by MS/MS the flagellar fragments tested, from 11168-O WT, 11168-GS WT and 11168-O 0466<sup>-</sup> grown in broth or on agar, were all identified as *C. jejuni* NCTC 11168 FlaA, the major flagellin protein, indicating that flagellin was present in all samples. It was impossible to tell whether the minor flagellin protein, FlaB, was also present, due to the high sequence similarity of the two flagellins (93% in 11168-GS WT); although there was no evidence of the presence of FlaB specific peptides in any of the samples. Tables 5.1 and 5.2 show the percentage of peptide coverage in all samples when analysed by CID and ECD respectively.

**Table 5.1. Peptide coverage of *C. jejuni* NCTC 11168 Flagellin analysed by CID MS/MS**

Sample	Protein identification	Coverage (%)
11168-O WT	Flagellin (flaA) [ <i>Campylobacter jejuni</i> NCTC 11168]	48
11168-GS WT	Flagellin (flaA) [ <i>Campylobacter jejuni</i> NCTC 11168]	52
11168-O 0466 <sup>-</sup> (Broth grown)	Flagellin (flaA) [ <i>Campylobacter jejuni</i> NCTC 11168]	49
11168-O 0466 <sup>-</sup> (Agar grown)	Flagellin (flaA) [ <i>Campylobacter jejuni</i> NCTC 11168]	43

**Table 5.2. Peptide coverage of *C. jejuni* NCTC 11168 Flagellin analysed by ECD MS/MS**

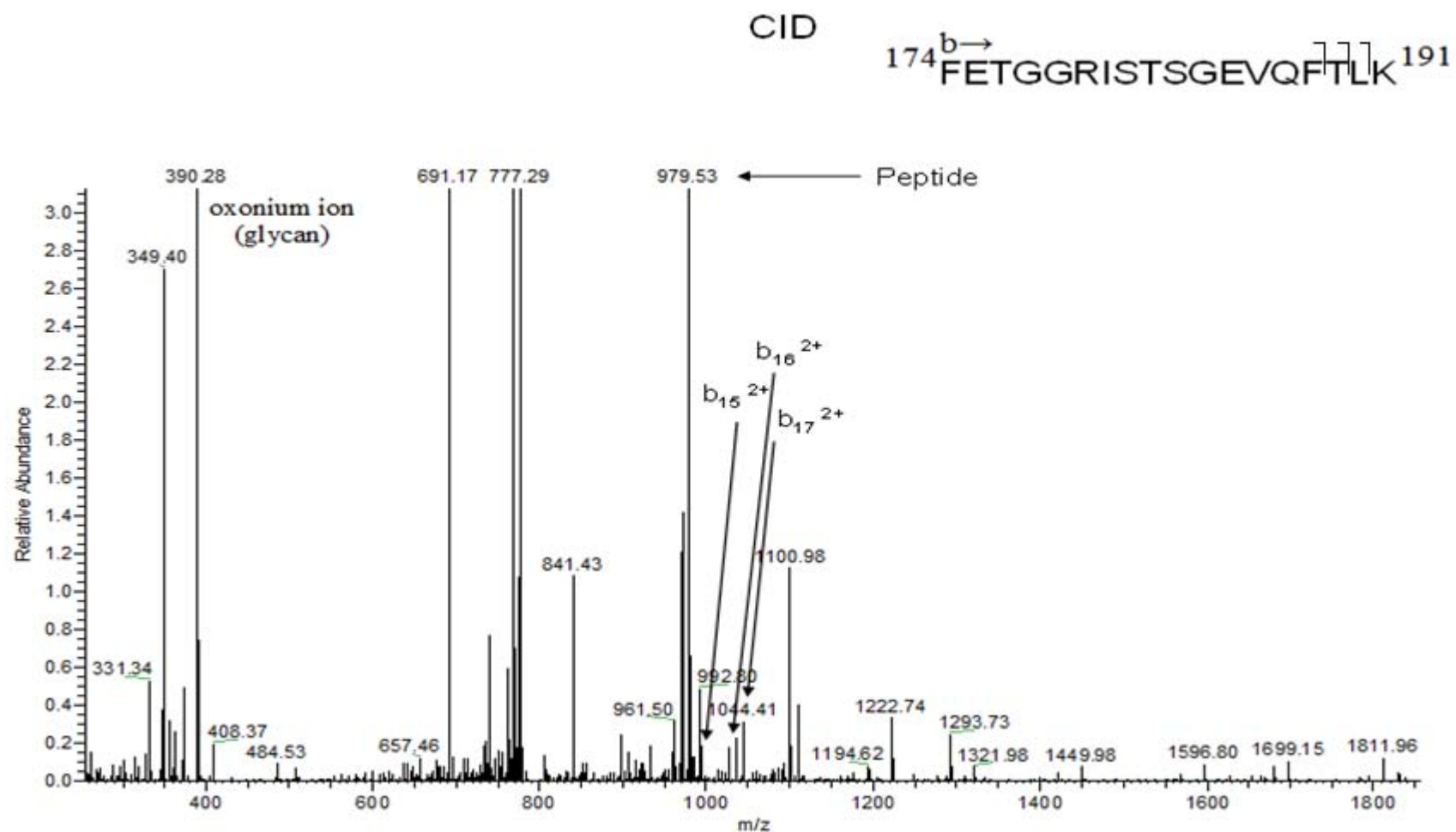
Sample	Protein identification	Coverage (%)
11168-O WT	Flagellin (flaA) [ <i>Campylobacter jejuni</i> NCTC 11168]	52
11168-GS WT	Flagellin (flaA) [ <i>Campylobacter jejuni</i> NCTC 11168]	34
11168-O 0466 <sup>-</sup> (Broth grown)	Flagellin (flaA) [ <i>Campylobacter jejuni</i> NCTC 11168]	43
11168-O 0466 <sup>-</sup> (Agar grown)	Flagellin (flaA) [ <i>Campylobacter jejuni</i> NCTC 11168]	41

When analysed by both the CID MS/MS and ECD MS/MS methods, no glycans were identified on flagellar peptides from 11168-GS WT or the 11168-O 0466<sup>-</sup> mutant, grown either on agar or in broth. Despite several repetitions of MS/MS analysis, using material from two separate flagellin isolations, no glycans could be indentified. However, one glycan, a di-*O*-methyl-glyceric acid derivative of acetamidino pseudaminic acid was identified in two places on 11168-O WT flagellin.

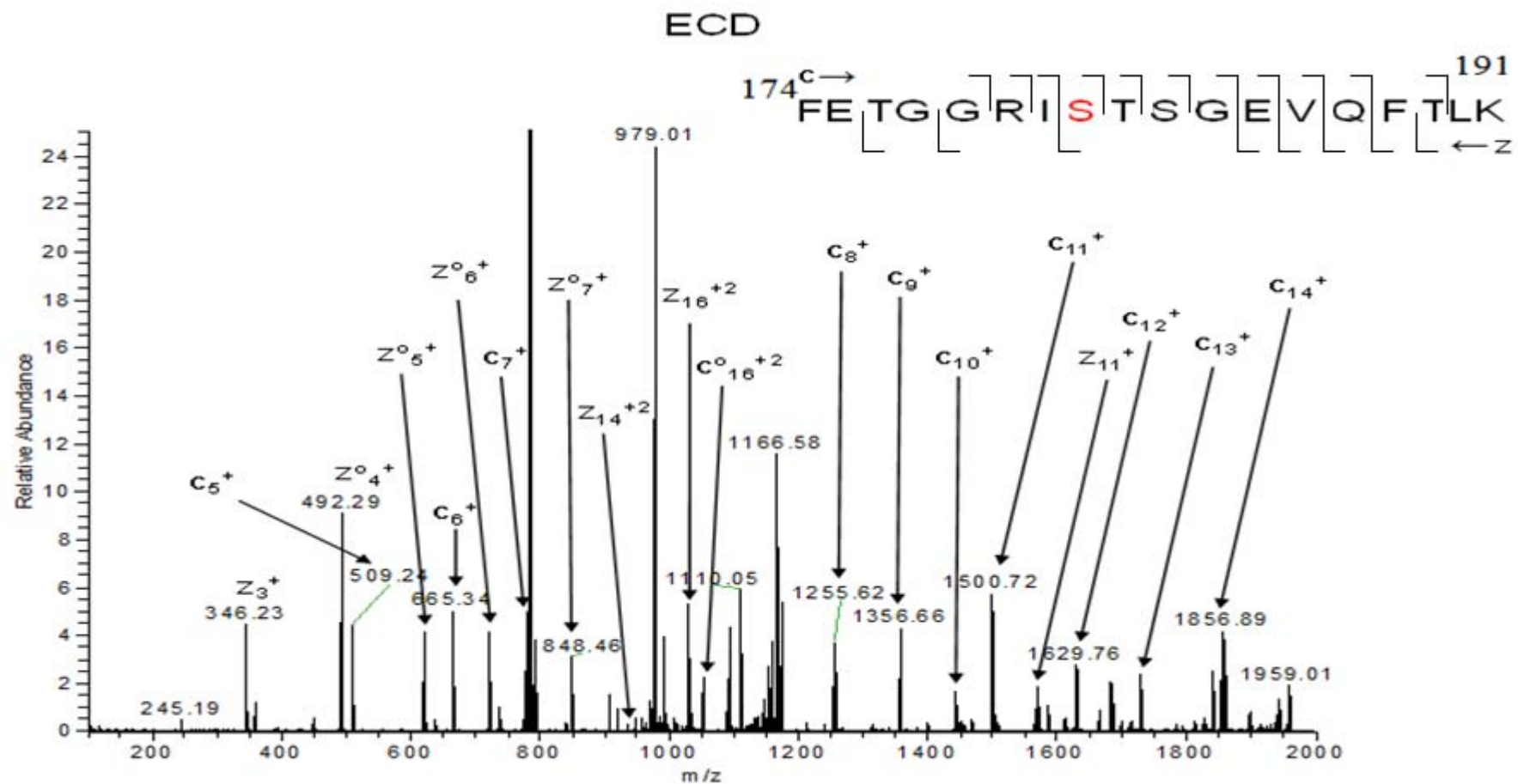
One of the modified peptides was 174- FETGGRISTSGEVQFTLK-191 which is located in the central region of FlaA; the serine residue bearing the modification is underlined. The glycan was identified as the dimethylglyceric acid derivative of acetamidino pseudaminic acid (PseAm), which was first identified as a major glycosylation of *C. jejuni* 11168 flagellin by Logan *et al.* (2009). Figure 5.4 shows the fragments obtained from CID of the FETGGRISTSGEVQFTLK peptide. As only three b-fragment ions and no y-fragment ions were identified, it is impossible to determine the sequence of this peptide from this data, nor can any information regarding the location of the glycan can be derived. The unfragmented glycan, which has been released from the glycopeptide is visible in the form of its oxonium ion, is visible at m/z 390.28. The intact doubly charged peptide is visible at m/z 979.53, indicating that the peptide backbone was not fragmented during CID, which is in agreement with the lack of fragment ions. Figure 5.5 shows the fragments generated by ECD of the FETGGRISTSGEVQFTLK peptide. The large number of C- and Z-fragment ions indicate that the peptide backbone has been fragmented and allows the peptide sequence to be determined. By comparing the experimental masses of the fragment ions obtained here to the values expected without glycosylation, it is possible to locate the glycosylated residue.



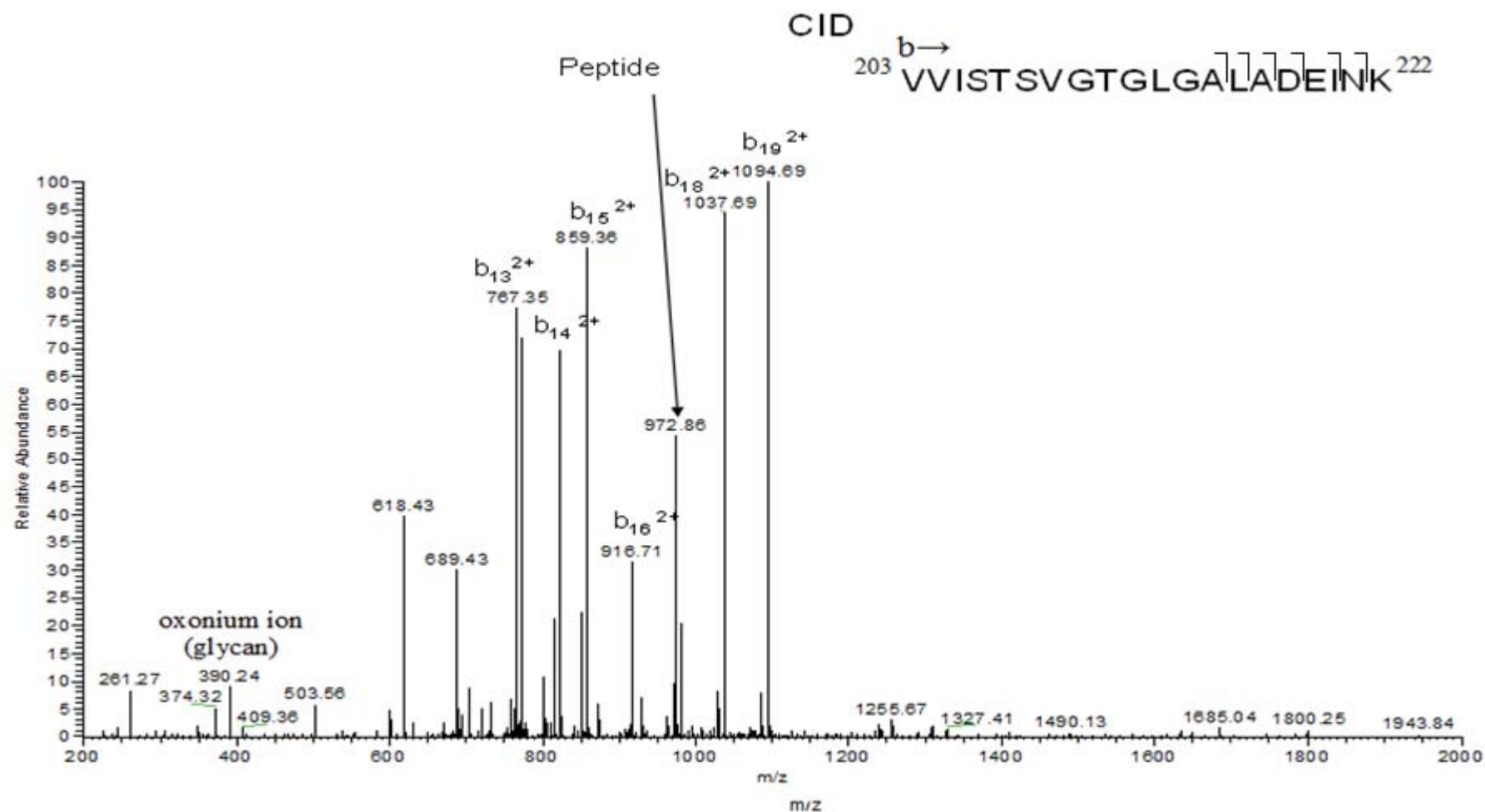
The second modified peptide identified in 11168-O WT flagellin was 203-VVISTSVGTGLGALADEINK-222; the amino acid residue bearing the modification is underlined. This peptide is also located in the central region of the *C. jejuni* 11168 FlaA protein and was modified with the same dimethylglyceric acid derivative of acetamidino pseudaminic acid as the first peptide. Again, too few b-fragment ions were generated by CID to deduce the peptide sequence or locate the glycosyl-modification, as shown in Figure 5.6. The intact peptide minus the glycan was identifiable, as was the oxonium ion of the intact glycan at  $m/z$  390.24. The ECD spectrum shows the large number of C- and Z-fragment ions which together allow the peptide sequence and the location of the glycosyl modification to be deduced, see Figure 5.7.



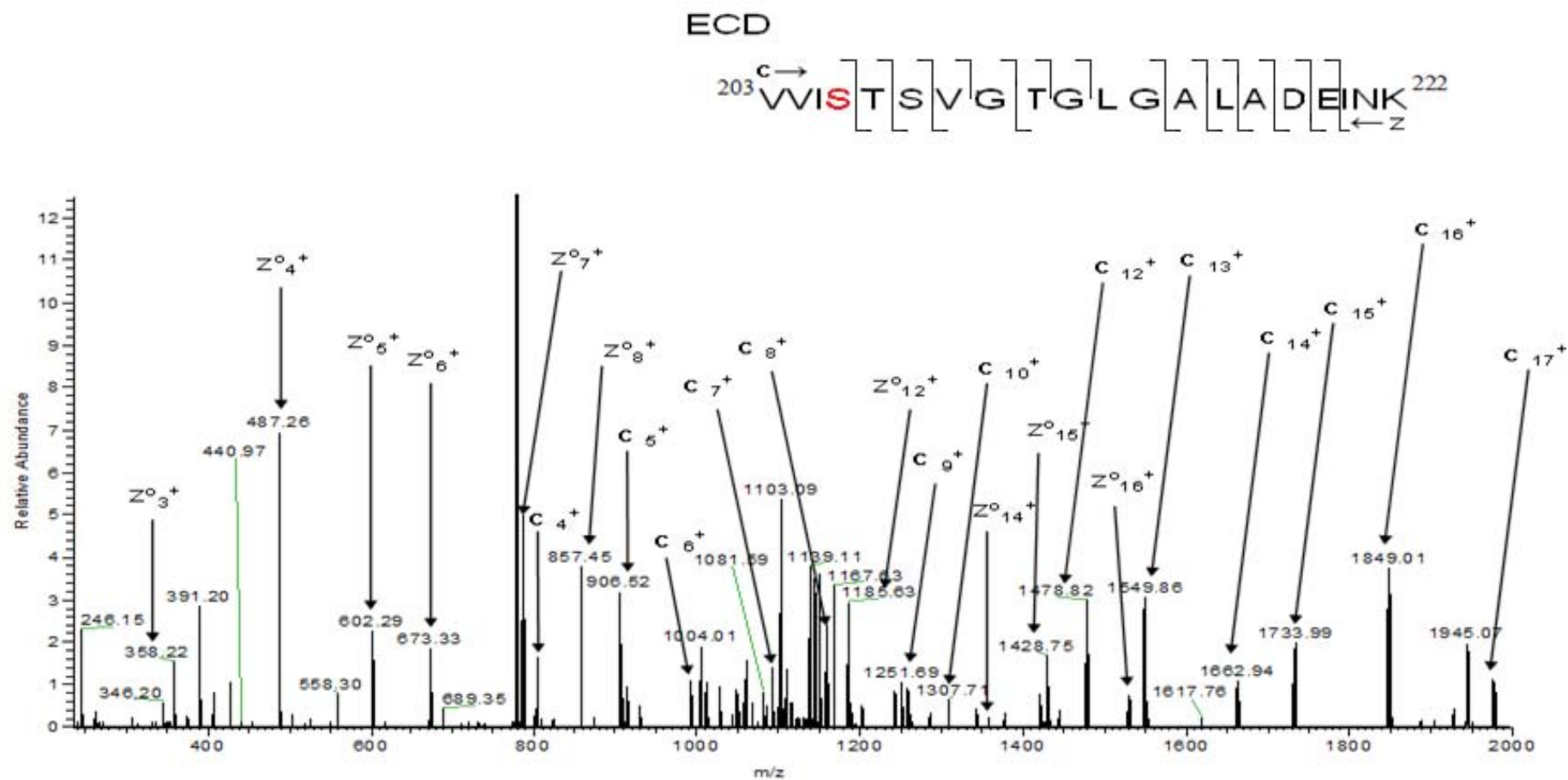
**Figure 5.4. CID fragments of the FETGGRISTSGEVQFTLK peptide.** The three b fragment ions arising from fragmentation of the peptide bonds are shown, as is the oxonium ion of the glycan residue at m/z 390.28 and the doubly charged peptide minus the glycan at m/z 979.53.



**Figure 5.5 ECD fragments of the FETGGRISTSGEVQFTLK peptide.** Showing the C- and Z-fragment ions arising from fragmentation of the peptide bonds, lines dividing the peptide sequence show the locations of the breakages of the peptide backbone which produced these ions. The peptide fragmentation allows the peptide sequence to be determined and the glycan to be located to the serine residue highlighted in red.



**Figure 5.6. CID fragments of the VVISTSVGTGLGALADEINK peptide.** The six b-fragment ions arising from fragmentation of the peptide bonds are shown, as is the glycan residue at  $m/z$  390.24 and the doubly charged peptide minus the glycan at  $m/z$  972.8.



**Figure 5.7. ECD fragments of the VVISTSVGTGLGALADEINK peptide.** Showing the C- and Z-fragment ions arising from fragmentation of the peptide bonds and the locations of peptide bond breakage. This allows the peptide sequence to be determined and the glycan to be located to the serine residue highlighted in red.

## 5.4 Discussion

### 5.4.1 Variation in flagellin glycosylation

Flagellar pieces isolated using a variation on the extensively used method of Power *et al.* (1994) were examined by SDS-PAGE (Figure 5.1). The 11168-O 0466<sup>-</sup> flagellin isolated from exponential phase broth grown cells gave a considerably less dense band than that of the same strain grown on agar. This reflects the difficulty of isolating sufficient amounts of flagellar pieces from this strain as, under these growth conditions, only short, truncated flagella are produced. Also apparent in Figure 5.2 is the breadth of the flagellin bands which are diffuse and not as sharp and compact as would be expected from SDS-PAGE of a single protein. It is possible that this is an artefact of the SDS-PAGE analysis or the flagellin isolation procedure; alternatively it could be indicative of variation in the mass of the flagellin isolated. The variation could potentially be due to natural variation in the types and locations of glycans applied to the flagellin proteins by the cell. Variation in flagellin mass due to gene and gene expression differences has been described (Thibault *et al.*, 2001; Turner and Penn, 2005); whether this type of can occur within a single population of cells is unclear. This variation could also be generated by the loss of flagellar glycans during the purification process or otherwise be due to natural variation in the types and locations of glycans applied to the flagellin proteins by the bacterial cell. When whole cell lysates were examined by SDS-PAGE, see Figure 5.3, the flagellin bands of both 11168-O WT and 11168-O 0466<sup>-</sup> appeared to be sharp and did not suggest that there was any large variation in flagellin glycosylation within these strains. This gel does however show the variation in mass between 11168-O WT and 11168-O 0466<sup>-</sup> flagellins. This suggests that any apparent variation in flagellin mass seen within a particular strain may actually be artefactual.

Studies which have used isoelectric focusing to characterise flagellins from *C. jejuni* wild-type strains (including 11168-GS WT) and defined mutants have provided some evidence of variation in flagellar glycans, by demonstrating the separation of flagellins into multiple ‘glycoforms’ in both wild-type and mutant flagellins (Doig *et al.*, 1996; Guerry *et al.*, 2006). However, the majority of these studies also use the method of flagellar isolation developed by Power *et al.* (1994), it therefore remains difficult to determine whether the presence of multiple glycoforms of flagellin is due to natural variation in the number and nature of the glycans attached to flagellin proteins or simply due to the loss of glycans during the isolation process.

The identification and location the flagellar glycans of *C. jejuni* 81-176 by Thibault *et al.* (2001) provides evidence to suggest that there is little natural variation in flagellin glycosylation as it was shown that, although there was a certain amount of micro-heterogeneity in the natures of the *O*-linked glycans attached to flagella, all of the 19 identified glycosylation sites were occupied on each flagellin monomer. Hence it is probable that the potential variation in flagellin mass seen here is due not to natural variation in flagellin glycosylation caused by alterations in the number and type of glycans applied to flagellin by the cell, but instead to the method of isolation used. This is also supported by evidence that at least a certain level of flagellin glycosylation is required for flagellar assembly, as mutants in certain PseAc biosynthesis genes which do not glycosylate flagellin do not produce flagella (Goon *et al.*, 2003; Guerry *et al.*, 1996).

#### 5.4.2 Mass spectrometry

The aim of this part of the study was to begin to map the type and locations of flagellar glycans present on the flagellins of the 11168-O WT, 11168-O 0466<sup>-</sup> mutant

and 11168-GS WT isolated from log-phase broth and 11168-O 0466<sup>-</sup> flagellin isolated from cells grown on agar. Any differences observed in the glycans present on the flagellins could be used to help to explain the differences in flagellin mass between the strains, as observed by SDS-PAGE, and potentially the presence of short, asymmetric flagella in the 11168-O 0466<sup>-</sup> mutant when grown on soft motility agar.

#### 5.4.2.1 Glycosylation of 11168-O WT flagellin

Analysis by mass spectrometry carried out as part of this work indicated that the major flagellin protein, FlaA, of *C. jejuni* 11168-O WT was glycosylated in two places with a dimethylglyceric acid modification of acetamidino pseudaminic acid (PseAm) which was recently discovered by Logan *et al.* (2009). Another novel glycan, a dimethylglyceric acid modification of pseudaminic acid (PseAc), was also identified by Logan *et al.* (2009) and the presence of other previously detailed flagellar glycans in the metabolome of *C. jejuni* 11168 was confirmed; these glycans included PseAc, PseAm and two derivatives of legionaminic acid. The two novel glycans were found to be the most common modifications present on *C. jejuni* 11168 flagellin.

Although no information concerning the overall level of glycosylation of *C. jejuni* 11168 flagellin or the locations glycans across the flagellin exists, the difference in FlaA mass when predicted from the amino acid sequence and observed by SDS-PAGE analysis suggests that slightly less than 10% of flagellin mass will consist of glycans. In *C. jejuni* 81-176, 10% of flagellin mass comprises carbohydrate, which corresponds to 19 glycans. Hence it is very probable that *C. jejuni* 11168 flagellin is glycosylated at considerably more locations than the two identified here, or those detailed by Logan *et al.* (2009).



It is unclear why no other glycans were identified by the MS/MS analysis carried out here; it is possible that the flagellar isolation process caused the loss of some flagellar glycans before MS/MS analysis was carried out. If specific modifications were particularly subject to loss during the isolation process, the fact that CID and ECD fragmentation was carried out on only the most abundant ions present in each scan, the top three ions in each scan were subjected to CID and only the most abundant ion from each scan was selected for ECD, could help to explain why these modifications were not detected.

The incomplete peptide coverage, 48% for CID MS/MS of 11168-O WT flagellin and 52% for ECD MS/MS of 11168-O WT flagellin, will also have contributed to the lack of flagellar glycans identified. Whilst peptides corresponding to those known to be glycosylated in *C. jejuni* 81-176 (Thibault *et al.*, 2001) were identified during this MS/MS analysis, several did not carry glycan residues. However not all of these corresponding glycosylated peptides were identified, with or without glycans, suggesting that could be present on peptides which were not identified in this work. Other reasons for the lack of glycans discovered could be more intrinsic to the samples analysed as glycopeptides can be subject to poor ionisation during the initial stages of MS/MS analysis, peptides which fail to ionise are eluted from the mass spectrometer prior to MS/MS analysis and hence are not detected. Low stoichiometric abundance of particular types of modifications can also prevent the identification of glycan modifications. The two novel dimethylglyceric acid modifications of PseAc and PseAm were identified by Logan *et al.* (2009) as the prevalent modifications of *C. jejuni* 11168 flagellin, the failure to detect other modifications, including PseAc, PseAm and legionaminic acid derivatives, could simply be due to the fact that these modifications were not present in sufficient amounts.

The multiple alignment shown in Figure 5.8 indicates that the locations of glycosylation are quite well conserved between *C. coli* VC167 and *C. jejuni* 81-176, although the actual types of glycan applied are not. Also evident is the level of sequence conservation between the flagellins of these strains, particularly at the N- and C-terminal regions, which are thought to be involved in flagellar subunit interactions and flagellar assembly (Yonekura *et al.*, 2003). The majority of glycans appear to be located in the central region of the flagellin, which is also the most diverse region in terms of sequence. Although, despite the differences in sequence, the relative locations of the glycan modifications appear to be well conserved.

It is impossible to determine whether glycosylation in *C. jejuni* 11168 also follows the pattern established from *C. jejuni* 81-176 and *C. coli* VC167 flagellin, until a more complete map of flagellar glycosylation in this strain is available. However, given the flagellin sequence similarities and the importance of flagellin glycosylation to flagellar filament formation (Goon *et al.*, 2003), it is probable that *C. jejuni* 11168 will show a similar pattern of glycosylation to *C. jejuni* 81-176 and *C. coli* VC167, although there is clearly significant variation in the nature of the glycans applied. The large flagellin glycosylation locus of *C. jejuni* 11168 contains approximately 50 genes in comparison to the 23 genes of *C. jejuni* 81-176 and 14 of *C. coli* VC167, suggesting that the modifications present on *C. jejuni* 11168 flagellin will be more diverse than those of other strains; the discovery of two novel modifications by Logan *et al.* (2009) supports this. As many genes in the *C. jejuni* 11168 flagellin glycosylation locus do not yet have defined functions, it is possible that yet more glycans remain to be discovered.

C.jejuni_11168	MGFRINTNVAALNAKANADLNSKSLDASLSRLSSGLRINSAADDASGMAIADSLRSQANT
C.jejuni_81176	MGFRINTNIGALNAHANSVNSNELDKSLRLSSGLRINSAADDASGMAIADSLRSQAAT
C.coli_VC167	-GFRINTNVAALNAKANSDLNSRALDQSLRLSSGLRINSAADDASGMAIADSLRSQANT
C.jejuni_11168	LGQAISNGNDALGILQTADKAMDEQLKILDITIKTKATQAAQDGQSLKTRTMLQADINRLM
C.jejuni_81176	LGQAINNNGNDAIGILQTADKAMDEQLKILDITIKTKATQAAQDGQSLKTRTMLQADINKLM
C.coli_VC167	LGQAISNGNDALGILQTADKAMDEQLKILDITIKTKATQAAQDGQSLKTRTMLQADINRLM
C.jejuni_11168	EELDNIAANTTSFNGKQLLSGNFINQEFQIGASSNQTVKATIGATQSSKIGLTRFETGGRI
C.jejuni_81176	EELDNIAANTTSFNGKQLLSGNFTNQEFQIGASSNQTVKATIGATQSSKIGVTRFETGAQS
C.coli_VC167	EELDNIAANTTSFNGKQLLSGGFTNQEFQIGSSSNQTIKASIGATQSSKIGVTRFETGSQS
C.jejuni_11168	TSGEVQFTLKNYNGIDDFQFQKVVISVSVGTGLGALADEINKNADKTGVRATFTVETRG
C.jejuni_81176	FTSGVVGLTIKNYNGIEDFKFDNVVISVSVGTGLGALAEINKSADKTGVRATYDVKTG
C.coli_VC167	FSSGTVGLTIKNYNGIEDFKFQNVVISVSVGTGLGALAEINRNADKTGIRATFDVKSQV
C.jejuni_11168	IAAVRAGATSDTFAINGVKIGKVDYKDGANGALVAAINSVKDTTGVEASIDANGQLLLT
C.jejuni_81176	VYAIKEGTTSDFAINGVVIGQINYKDGDNNGQLVSAINAVKDTTGQASKDENGKLVLT
C.coli_VC167	AYAIAKAGNTSDFAINGVVIGKVDYSDGDENGSLISAINAVKDTTGQASKDENGKLVLT
C.jejuni_11168	SREGRGIKIDGNIGGGAFINADMKENYGRSLSLVKNDGKDILISGSNLSSAGFGATQFISQ
C.jejuni_81176	SADGRGIKITGDIGVGSILANQKENYGRSLSLVKNDGRDINISGTNLSAIGMGTTDMISQ
C.coli_VC167	SADGRGIKITGSIGVGAGIL--HTENYGRSLSLVKNDGRDINISGTLSAIGMGATDMISQ
C.jejuni_11168	ASVSLRESKGQIDANIADAMGFGSANKG----VVLGGYSSVSAYMSSAGSGFSSSGSGYSV
C.jejuni_81176	SSVSLRESKGQISATNADAMGFNSYKGGG-KFVFQNVSSISAFMSAQSGFSRSGSGSV
C.coli_VC167	SSVSLRESKGQISAANADAMGFNAYNGGAKQIIFA--SSIAGFMSQAGSGFSAGSGFSV
C.jejuni_11168	GSGKNYSTGFANAIAI-SAASQLSTVYNVSAGSGFSSGSTLSQFATMKTTAFGVKDETAG
C.jejuni_81176	GSGKNLSVGLSQGIQIISSAASMSNTYVVSAGSGFSGSGNSQFAALKTTAANTTDETAG
C.coli_VC167	GSGKNYSAILSAIQIVSSAASISSTYVVSSTGSGFSAGSGNSQFAALRISTVSAHDETAG
C.jejuni_11168	VTTLKGAMAVMDIAETAITNLDQIRADIGSVQNQVTSTINNITVTQVNVKAAESQIRDVD
C.jejuni_81176	VTTLKGAMAVMDIAETAITNLDQIRADIGSVQNQLQVTINNITVTQVNVKAAESTIRDVD
C.coli_VC167	VTTLKGAMAVMDIAETAITNLDQIRADIGSVQNQITSTINNITVTQVNVKSAESQIRDVD
C.jejuni_11168	FAAESANYSKANILAQSGSYAMAQANSVQQNVLRLLQ
C.jejuni_81176	FAESANFSKYNILAQSGSYAMSQANAVQQNVLRLLQ
C.coli_VC167	FAESANYSKANILAQSGSYAMAQANSQQNVLRLLQ

**Figure 5.8. Alignment of FlaA from *C. jejuni* NCTC 11168, *C. jejuni* 81-176 and *C. coli* VC167.** Those residues modified with glycans are indicated. *C. jejuni* 81-176 modifications are taken from Thibault *et al.* (2001), *C. coli* VC167 modifications are taken from Logan *et al.* (2002). *C. jejuni* 11168 modifications shown are those identified in this work. (The sequences were obtained from CampyDB at <http://xbase.bham.ac.uk/campydb/> and aligned using the ClustalW2 tool available from EBI at <http://www.ebi.ac.uk/Tools/clustalw2/index.html>, accessed August, 2009).

#### 5.4.2.2 Flagellin glycosylation in *C. jejuni* 11168-O 0466<sup>-</sup> and 11168-GS WT

No flagellar glycans were identified on flagellin from 11168-GS WT or 11168-O 0466<sup>-</sup>. The aim of this part of the analysis was to identify and ‘map’ the locations and types of glycans present on flagellin from these strains. This information would then be compared to the map of glycosylation for 11168-O WT flagellin to begin to elucidate some of the observed differences between these strains. The flagellin of 11168-GS WT has a lower mass than that of 11168-O WT (Turner and Penn, 2005) which is likely to be due to a decrease in the amount of glycosylation of flagellin in 11168-GS WT; although it is probable that this flagellin is still glycosylated to some degree as glycosylation appears to be necessary for flagellar filament formation (Goon *et al.*, 2003). Likewise the flagellin of 11168-O 0466<sup>-</sup> also has a lower mass than that of 11168-O WT. These ‘maps’ could also have been used to determine whether differences in flagellin glycosylation were responsible for the inability of 11168-O 0466<sup>-</sup> cells, growing at fast rates, to form full length flagella.

Potential reasons for the failure of MS/MS analysis to locate flagellar glycans could include many of those reasons detailed above (Section 5.4.2.1), such as the possible loss of glycans prior to MS/MS analysis, incomplete peptide coverage and poor peptide ionisation, which can affect MS/MS of glycopeptides (Alley *et al.*, 2009). The possibility of low abundance of particular modifications could also be responsible, as the MS/MS method used here to locate the flagellar modifications, ECD, is known to be less sensitive than other MS/MS methods (Zubarev, 2004). It is also possible that the dimethylglyceric acid modifications of acetamidino pseudaminic acid located on 11168-O WT flagellin are not present on the flagellin of the other strains. Whether this is the case or not is difficult to predict as the biosynthetic process of the dimethylglyceric acid

PseAm is not known and so it is not possible to identify which changes in gene expression could be responsible for loss of these glycans.

## 5.5 Conclusions and future work

From this work it is possible to conclude that 11168-O WT flagellin is glycosylated in at least two, and probably more, locations and that one of the glycans is present as a dimethylglyceric acid derivative of acetamidino pseudaminic acid. This information agrees with the published data (Logan *et al.*, 2009), but is not as complete. The failure of MS/MS to generate glycosylation mapping information for 11168-GS WT and 11168-O 0466<sup>-</sup>, from both growth conditions, makes it impossible to link changes in flagellin glycosylation in these strains to observed changes in flagellin mass, flagellar structure and motility. It is also impossible to link any potential glycosylation changes to alterations in gene complement or expression. There is, from other work, evidence to suggest that the 11168-O 0466<sup>-</sup> could be lacking flagellar glycans including LegAm and PseAm (Howard *et al.*, 2009; Guerry *et al.*, 2006). However there is also evidence to suggest that flagellin glycosylation is necessary for flagellar formation and function in *Campylobacter* (Goon *et al.*, 2003; Guerry *et al.*, 1996), and as the strains used in this study all produce flagella, although of varying lengths, they are likely to be glycosylated. Although, as this work was carried out in several different strains of *Campylobacter*, it is necessary to more fully map the flagellar glycans present in these strains before attempting to draw comparisons.

To that end, other approaches to the task of identifying and locating flagellar glycans could involve the focused-metabolomics based approach adopted during several studies of *Campylobacter* flagellin (Logan *et al.*, 2009; McNally *et al.*, 2006a and McNally *et al.*, 2007). This approach has been very successful in identifying the glycans

themselves, although their mapping to particular amino acid residues still relies on mass spectrometry based approaches, which have had varied levels of success. Another approach could be to attempt to improve the quality of the starting material by improving the method used for the isolation and enrichment of flagellar pieces, as it is possible that glycans are being lost from flagellin at this stage. Although many isolation methods rely on the generation of shear-forces within a sample to separate pieces of flagella from bacterial cells, other methods, such as glycine extraction, are available (Nilsson *et al.*, 2000).

## Chapter VI: General Discussion

Protein glycosylation was once considered to be a strictly eukaryotic phenomenon; however, there is now an increasing awareness of prokaryotic glycoproteins, particularly of surface proteins, including flagella. Flagellin was the first glycoprotein identified in *Campylobacter* and remains the best characterised. The majority of research in the area of flagellin glycosylation in *Campylobacter* has focused on identifying the glycans applied to flagella, the functions of the genes responsible for glycan biosynthesis and attachment, and the elucidation of the pathways of glycan biosynthesis (Guerry *et al.*, 1996; McNally *et al.*, 2006a; Thibault *et al.* 2001, and others). This has led to the identification of a large and growing array of glycans which decorate *Campylobacter* flagellin and many groups of genes involved in glycan biosynthesis including the *pse*, pseudaminic acid and related derivative biosynthetic genes and the *ptm* genes, responsible for legionaminic acid synthesis.

The regulation of flagellin glycosylation is not well understood. Given that at least a certain level of glycosylation is required for filament formation (Goon *et al.*, 2003), it is possible that at least some flagellin glycosylation genes will be co-ordinately regulated with the flagellar structural genes. In support of this hypothesis,  $\sigma^{28}$  has been implicated in the regulation of a small number of flagellin glycosylation genes (Carrillo *et al.*, 2004). However, the mechanisms of regulation for the majority of flagellar glycosylation genes remain unclear. This study investigates the action of the nitrosative stress response regulator in *C. jejuni*, NssR, upon a subset of 17 flagellin glycosylation genes, the FMR. NssR was found, by chromatin immunoprecipitation analysis, to bind to at least four sites within the FMR. Analysis of the transcriptional organisation of the FMR identified six possible transcripts

beginning in the region, three of which potentially started ahead of a gene in which NssR binding had been identified. Hence it is likely that NssR binds to the promoter regions of a certain subset of FMR genes to modulate gene expression in this region. Further work would be required to confirm this as it was not possible to identify either the transcript start points of the genes involved or the NssR binding motifs within the FMR.

The deletion of *nssR* causes changes to the flagella of the mutant which include a decrease in flagellin mass, probably associated with uncharacterised changes in glycosylation. The motility of the  $\Delta nssR$  mutant is also altered it demonstrates a significant decrease in speed in comparison to the wild-type and loses the darting motility typical of *Campylobacter*, acquiring a more circular, spinning type of motion. When the flagella of  $\Delta nssR$  mutant cells, taken from a rapidly dividing culture, were examined by EM, short, asymmetric flagella were observed. It is possible that the inability of this mutant to form full length flagella could be due to a defect in flagellar biosynthesis, potentially caused by changes in glycosylation. Attempts to create a complete map of the flagellar glycans present in the original strain wild-type and mutant by mass spectrometry were unsuccessful.

It remains unclear why NssR is involved in the regulation of a group of genes associated with flagellin glycosylation. NssR is a member of the Crp-Fnr superfamily of transcriptional regulators which are involved in regulating the response to a broad spectrum of intracellular and exogenous signals, including oxidative and nitrosative stress, nitric oxide, carbon monoxide, 2-oxoglutarate, anoxia, cAMP, redox state and temperature (Korner *et al.*, 2003). These regulators can modulate the activity of a large range of genes, it is possible that NssR is acting in response to an unknown signal to modulate expression of the FMR and consequently flagellin glycosylation and so potentially motility and flagella filament formation. Oxygen, by a largely unknown mechanism, together with NssR, has been found to



positively regulate Cgb, a globin protein involved in NO detoxification, which was previously thought to be regulated by NssR alone (Monk *et al.*, 2008). It is also possible that oxygen availability is a factor which influences NssR regulated gene expression; the repertoire of signals which modulate *nssR* expression remains to be determined.

Further work is required to better understand the action of NssR, not only in the FMR but across the entire *C. jejuni* genome. The chromatin immunoprecipitation method developed in this work could be used to isolate DNA which could then be sequenced using high-throughput DNA sequencing technologies; this would provide a genome-wide map of NssR binding. Identification of the glycans present on wild-type and mutant flagella could be very useful in explaining some of the phenotypic differences between these strains, and in identification of those genes involved in glycan biosynthesis. The mass spectrometry approach used here had some success in this area, but to generate a complete map of flagellin glycosylation, improvements in the method of flagella isolation would be needed. A metabolomic analysis, to identify flagellar glycan precursors present in the  $\Delta nssR$  mutant, could also be useful to begin to determine the pathways of glycan synthesis in this strain.

## Appendix I - Sequence of the pDex\_Fcc plasmid

```

0  GACGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAATAATGGTTT
60  CTTAGACGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTT
120  TCTAAATACATTCAAAATATGTATCCGCTCATGAGACAATAACCCCTGATAAATGCTTCAAT
180  AATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCTTTT
240  TTGCGGCATTTTGCCTTCCTGTTTTTGTCTACCCAGAAACGCTGGTGAAAGTAAAAGATG
300  CTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAAGA
360  TCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGC
420  TATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGTGCGCCGATAC
480  ACTATTCTCAGAATGACTTGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATG
540  GCATGACAGTAAAGAGAATTATGTCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCA
600  ACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGG
660  GGGATCATGTAACCTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACG
720  ACGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACTATTAAC TG
780  GCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGATAAAG
840  TTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGTTTATTGCTGATAAATCTG
900  GAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGACGCACTGGGGCCAGATGGTAAGCCCT
960  CCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGAC
1020 AGATCGCTGAGATAGGTGCCCTCACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTACT
1080 CATATATACTTTAGATTGATTTAAACTTCATTTTTTAATTTAAAGGATCTAGGTGAAGA
1140 TCCTTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCTGTTCCACTGAGCGT
1200 CAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCT
1260 GCTGCTTGCAACAAAAAAACACCCGCTACCGCGGTGGTTTGTGTTGCCGGATCAAGAGC
1320 TACCAACTCTTTTCCGAAGGTAACCTGGCTTCAGCAGAGCGCAGATACCAATACTGTCC
1380 TTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCTACATACC
1440 TCGCTCTGCTAATCCTGTTACCGATGGCTGCTGCCAGTGGCGATAAAGTCGTGTCTTACC
1500 GGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTGCGGCTGAACGGGGGGTT
1560 CGTGCACACAGCCAGCTTGAGAGCAACGACCTACACCGAACTGAGATACCTACAGCGTG
1620 AGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGCGGACAGGTATCCGGTAAGCG
1680 GCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGAAACGCCCTGGTATCTTT
1740 ATAGTCCTGTGCGGTTTTGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAG
1800 GGGGGCGGAGCTATGGAAAAACGCCAGCAACGCGGCCTTTTTTACGGTTCCTGGCCTTTT
1860 GCTGGCCTTTTGCTCACATGTTCTTTCTGCGTTATCCCCTGATTCTGTGGATAACCGTA
1920 TTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGAGCCGAACTGAGCGAGCGCAGCGAGT
1980 CAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAAACCGCTCTCCCCGCGCTTGGC
2040 CGATTCATTAATGTCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCA
2100 ACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAAGCTTTACACTTTATGCTTC
2160 CGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTACACAGGAAACAGCTATG
2220 ACCATGATTACGCCAAGCTCTAGGGATAACAGGGTAATCGATGAATTCAGCTTGGATCC
2280 CGGGTACCGACTACAAAGACCATGACGGTGATTATAAAGATCATGATATCGACTACAAAG
2340 ATGACGACGATAAATAGTAATGTAGGCTGGAGCTGCTTCGAAGTTCTTATCTTTCTAGA
2400 GCTGCTCGGCGGTGTTCTTTTCCAAGTTAATTGCGTGATATAGATTGAAAAGTGGATAGA
2460 TTTATGATAAGTGGATAGATTTATGATATATAATGAGTTaTCAACAAATCGGAATTTACGG
2520 AGGATAAATGATGCAATTCAAAAGATTGATATAAATAATTTGGACACGAAAAGAGTATTT
2580 CGACCACTATTTTGGCAATACGCCCTGCACATATAGTATGACGGTAAAACCTCGATATTTT
2640 TAAGTTGAAAAAGGATGGAAAAAAGTTATAACCAACTCTTTTATATGGAGTTACAACGAT
2700 CATCAATCGACATGAAGAGTTTACGACCGCATTAGATGAAAACGGACAGGTAGGCGTTTTT
2760 TTCAGAAATGCTGCCTTGCTACACAGTTTTTTCATAAGGAACTGAAACCTTTTCGAGTAT
2820 TTGGACTGAGTTTACAGCAGACTATACTGAGTTTCTTCAGAACTATCAAAAGGATATAGA
2880 CGCTTTTGGTGAACGAATGGGAATGTCCGCAAGCCTAATCCTCCGGAACACCTTTCCC
2940 TGTTTCTATGATACCGTGGACAAGCTTTGAAGGCTTTAACTTAAATCTAAAAAAGGATA
3000 TGACTATCTACTGCCGATATTTACGTTTGGGAAGTATATGAGGAGGGCGGAAAATACTA
3060 TATTCCCTTATCGATTCAAGTGCATCATGCCGTTTGTGACGGCTTTTCATGTTTGCCGTTT
3120 TTTGCTGAATTAACAAGACTTGCTGAATAAATAAAATCCAGTTTGTGCGCACTGATAAAA
3180 ACCGTTTAGGAACTAAAGGGCGCAGCTCGAGCTCATATGCTAGCGTCGACTAGTAGGGAT
3240 AACAGGGTAATGAGCTTGGCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCCT
3300 GCGGTTACCCAACTTAATCGCCTTGCAGCAATCCCCCTTTCGCCAGCTGGCGTAATAGC
3360 GAAGAGGCCCCCACCAGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGCGA
3420 GCTTGGCTGTTTGGCGGATGAGAGAAGATTTTCAGCCTGATACAGATTAAATCAGAACG
3480 CAGAAGCGGTCTGATAAAACAGAATTTGCCTGGCGGCAGTAGCGCGGTGGTCCCACCTGA

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3540 CCCCATGCCGA<sup>ACT</sup>CAGAAGTGA<sup>AAC</sup>GCCGTAGCGCCGATGGTAGTGTGGGGTCTCCCCA  
3600 TGCGAGAGTAGGGA<sup>ACT</sup>GCCAGGCATCAAAT<sup>AAAA</sup>CGAAAGGCTCAGTCGAAAGACTGGG  
3660 CCTTTCGTTTATCTGTTGTTTGTCTGGTGA<sup>AC</sup>GCCTCTCTGAGTAGGACAAATCCGCCGG  
3720 GAGCGGATTTGAACGTTGCGAAGCAACGGCCCGGAGGGTGGCGGGCAGGA<sup>CG</sup>CCCCGCCAT  
3780 AAAC<sup>T</sup>GCCAGGCATCAAATTAAGCAGAAGGCCATCCTGACGGATGGCCTTTTGTGCTTTC  
3840 TACAAACTCTTTTGTATTATTTTCTAAATACATTCAAATATGCATGCGCCTGATGCGGT  
3900 ATTTTCTCCTTACGCATATCGACATCCGCCCTCACCGCCAGGAACGCAACCGCAGCCTCA  
3960 TCACGCCGGCGCTTCTTGGCCGCGCGGGATTCAACCCACTCGGCCAGCTCGTCGGTGTAG  
4020 CTCTTTGGCATCGTCTCTCGCCTGTCCCCCTCAGTTCAGTAATTTCTGCA<sup>TT</sup>TGCTGTT  
4080 TCCAGTCGGTAGATATTCCACAAAACAGCAGGGAAGCAGCGCTTTTCCGCTGCATAACCC  
4140 TGCTTCGGGGTCATTATAGCGATTTTTTTCGGTATATCCATCCTTTTTCGCACGATATACA  
4200 GGATTTTGC<sup>CA</sup>AGGGTTCGTGTAGACTTTCTTGGTGTATCCAACGGCGTCAGCCGGGC  
4260 AGGATAGGTGAAGTAGGCC<sup>CA</sup>CCCGCAGCGGGTGTCTTCTTCACTGTCCCTTATTTCG  
4320 CACCTGGCGGTGCTCAACGGGAATCCTGCTCTGCGAGGCTGGCCGGCTACCGCCGGCGTA  
4380 ACAGATGAGGGCAAGCGGATGGCTGATGAAACCAAGCCAAC<sup>CA</sup>GGAAGGGCAGCCACCT  
4440 ATCAAGGTGTACTGCCTTCCAGACGAACGAAGAGCGATTGAGGAAAAGGCGGCGCGGCC  
4500 GGCATGAGCCTGTGCGCCTACCTGCTGGCCGTGCGCCAGGGCTACAAAATCACGGGCGTC  
4560 GTGGACTATGAGCAGTCCGCGAGCTGGCCCGCATCAATGGCGACCTGGGCGCGCTGGGC  
4620 GGCCTGCTGA<sup>AA</sup>ACTCTGGCTCACCGACGACCGCGCACGGCGCGGTTCGGTGATGCCACG  
4680 ATCCTCGCCCTGCTGGCGAAGATCGACTCTAGCTAGAGGATCGATCCTTTTAA<sup>CC</sup>CATC  
4740 ACATATACCTGCCGTTCACTATTATTTAGTGAAATGAGATATTATGATATTTTCTGAATT  
4800 GTGATTAAAAAGGCAACTTTATGCCCATGCAACAGAACTATAAAAAATACAGAGAATGA  
4860 AAAGAAACAGATAGATTTTTTAGTTCTTTAGGCCCGTAGTCTGCA<sup>AA</sup>TCTTTTATGATT  
4920 TTCTATCA<sup>AA</sup>CAAAAGAGGAAAATAGACCAGTTGCAATCCAACGAGAGTCTAATAGAAT  
4980 GAGGTCGAAAAGTAAATCGCGCGGGTTTGTACTGATAAAGCAGGCAAGACCTAA<sup>AT</sup>GT  
5040 GTAAAGGGCA<sup>AA</sup>AGTGTATACTTTGGCGTCA<sup>CC</sup>CCTACATATTTTAGGTCTTTTTTTATT  
5100 GTGCGTAAC<sup>T</sup>ACTTGCCATCTTCAAACAGGAGGGCTGGAAGAAGCAGACCGCTAACACA  
5160 GTACATAAAAAAGGAGACATGAACGATGAACATCA<sup>AA</sup>AAAGTTTGCAAAACAAGCAACAGT  
5220 ATTAACCTTTACTACCGCACTGCTGGCAGGAGGCGCAACTCAAGCGTTTGCGAAAGAAAC  
5280 GAACCAAAAGCCATATAAGGAAACATACGGCATTTC<sup>CA</sup>TATTACACGCCATGATATGCT  
5340 GCA<sup>AA</sup>TCCCTGAACAGCAAAAAATGAAAAATATCAAGTCTCTGAGTTCGATTCTGTCAC  
5400 AATTAAAAATATCTCTCTGCA<sup>AA</sup>AGGCCTGGACGTTGGGACAGCTGGCCATTACAAAA  
5460 CGCTGACGGCACTGTCGCA<sup>AA</sup>CTATCACGGCTACCACATCGTCTTTGCATTAGCCGGAGA  
5520 TCCTAAAAATGCGGATGACACATCGATTTACATGTTCTATCA<sup>AA</sup>AAAGTCGGCGAAACTTC  
5580 TATTGACAGCTGGAAAAACGCTGGCCGCGTCTTTAAAGACAGCGACAAATTCGATGCA<sup>AA</sup>  
5640 TGATTCTATCTTAAAGACCA<sup>AA</sup>ACACAAGAATGGTCAGGTTCAAGCCACATTTACATCTGA  
5700 CGGAAAAATCCGTTTATTCTA<sup>CA</sup>CTGATTTCTCCGGTAAACATTACGGCA<sup>AA</sup>ACAAACACT  
5760 GACAAC<sup>T</sup>GCA<sup>CA</sup>AGTTAACGTATCAGCATCAGACAGCTCTTTGAACATCAACGGTGTAGA  
5820 GGATTATAAA<sup>T</sup>CAATCTTTGACGGTGACGGAAAAACGTATCA<sup>AA</sup>ATGTACAGCAGTTCAT  
5880 CGATGAAGGCA<sup>ACT</sup>ACAGCTCAGGCGACAACCATACGCTGAGAGATCCTCACTACGTAGA  
5940 AGATAAAGGCCACA<sup>AA</sup>TACTTAGTATTTGAAGCAACACTGGAAC<sup>T</sup>GAAAGATGGCTACCA  
6000 AGGCGAAGAA<sup>T</sup>CTTTATTTAA<sup>CA</sup>AAAGCATACTATGGCA<sup>AA</sup>AGCACATCATCTCTCCGTCA  
6060 AGAAAGTCA<sup>AA</sup>AACTTCTGCA<sup>AA</sup>AGCGATAAAAAACGCACGGCTGAGTTAGCA<sup>AA</sup>ACGGCGC  
6120 TCTCGGTATGATTGAGCTAA<sup>AC</sup>GATGATTACACTGAAAAAGTGATGAAACCGCTGAT  
6180 TGCATCTAAC<sup>CA</sup>GTAACAGATGAAATTGAACGCGGAACGCTCTTTAA<sup>AA</sup>TGAACGGCAA  
6240 ATGGTATCTGTCACTGACTCCCGCGGATCA<sup>AA</sup>AAATGACGATTGACGGCATTACGTCTAA  
6300 CGATATTTACATGCTTGGTTATGTTTCTAATTCTTTAACTGGCCCATACAAGCCGCTGAA  
6360 CAA<sup>AA</sup>CTGGCCTTGTTGTTAA<sup>AA</sup>ATGGATCTTGATCCTAACGATGTAACCTTTACTTACTC  
6420 ACACTTCGCTGTACCTCAAGCGAAAGGAAACAATGTCGTGATTACAAGCTATATGACAAA  
6480 CAGAGGATTCTACGCAGACA<sup>AA</sup>CAATCAACGTTTGC<sup>CC</sup>TAGCTTCCTGCTGAACATCAA  
6540 AGGCAAGAAAA<sup>CA</sup>TCTGTTGTCAAAGACAGCATCCTTGAAC<sup>AA</sup>AGGACAATTAACAGTTAA  
6600 CAAATAAAAA<sup>CG</sup>CAAAAGAAAATGCCGATTATGGTGCAC<sup>T</sup>CTCAGTACAATCTGCTCTGA  
6660 TGCCGCATAGTTAAGCCAGCCCCGACACCCGCCAACACCCGCTGACGCGCCCTGACGGGC  
6720 TTGTCTGCTCCCGGCATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGTG  
6780 TCAGAGGTTTT<sup>CA</sup>CCGTCATCACCGAAACGCGCGA

## Appendix II - Buffers and Solutions

### Section 2.20.1. SDS-PAGE reagents

#### 2 x Resolving gels (12.5% acrylamide)

40% Acrylamide mix (Sigma, Gillingham, UK)	3.0 ml
1.5M Tris (pH 8.8)	2.5 ml
Sterile distilled water	4.4 ml
10% SDS	100 $\mu$ l
10% Ammonium Persulphate	50 $\mu$ l
TEMED	5 $\mu$ l

#### 2 x Stacking gels (4.0% acrylamide)

40% Acrylamide mix (Sigma, Gillingham, UK)	490 $\mu$ l
0.5M Tris (pH 6.8)	1.25 ml
Sterile distilled water	3.2 ml
10% SDS	50 $\mu$ l
10% Ammonium Persulphate	50 $\mu$ l
TEMED	5 $\mu$ l

#### Sample buffer

Laemmli sample buffer (BioRad, Hemel Hempstead, UK)	950 $\mu$ l
B-mercaptoethanol (Sigma, Gillingham, UK)	50 $\mu$ l

#### 10 x SDS-PAGE running buffer

Tris-HCl	30.3 g.l <sup>-1</sup>
Glycine	144 g.l <sup>-1</sup>
SDS	10 g.l <sup>-1</sup>
Sterile distilled water	to 1l

Coomassie stain

Coomassie blue R-25	0.1% (w/v)
Methanol	45% (v/v)
Glacial acetic acid	10% (v/v)
Sterile distilled water	to 100%

Destaining solution

Methanol	200 ml
Acetic acid	75 ml
Sterile distilled water	725 ml

**Section 3.2.8 - Purification of the NssR protein.**8 x binding buffer (pH 7.9)

4M NaCl
160mM Tris-HCl
40mM Imidazole

1 x wash buffer (pH 7.9)

0.5M NaCl
20mM Tris-HCl
60mM Imidazole

1 x elute buffer (pH 7.9)

0.5M NaCl
20mM Tris-HCl
1M Imidazole

**Section 3.2.10 - Western Blotting**Western blot running buffer

Glycine	14.4 g
Tris	3.0 g
Methanol	200 ml
Sterile distilled water	to 1l

10 x Tris buffered/saline (TBS, pH 7.6)

Trizma HCl	24.23 g
NaCl	80.06 g
Sterile distilled water	to 1l

1 x Tris buffered saline tween 20 buffer (TBST)

10 x TBS	100 ml
Tween 20	1 ml
Sterile distilled water	to 1l

Blocking solution

Fat-free dried milk powder	5 g
1 x TBST	to 100 ml

**Section 3.2.12 Chromatin immunoprecipitation (ChIP).**Lysis buffer

10mM Tris (pH 8.0)	
20% Sucrose	
50mM NaCl	
10mM EDTA (pH 8.0)	
Lysozyme to final concentration (20 mg.ml <sup>-1</sup> ) as needed	

IP buffer

50mM HEPES-KOH (pH 7.5)

150mM NaCl

1mM EDTA

1% Triton X-100

0.1% Sodium deoxycholate

0.1% SDS

IP salt buffer

50mM HEPES-KOH (pH 7.5)

500mM NaCl

1mM EDTA

1% Triton X-100

0.1% Sodium deoxycholate

0.1% SDS

Wash buffer

10mM Tris HCl (pH 8.0)

250mM LiCl

1mM EDTA

0.5% Nonidet P-40

0.5% Sodium deoxycholate

Elution buffer100mM NaHCO<sub>3</sub> (pH 9.0)

1.0% SDS

TBS buffer (pH 7.6)

10mM Tris HCl

150mM NaCl

**Section 3.2.13.4 - Electrophoretic mobility shift assay**10 x Binding buffer

1M Potassium Glutamate

10mM EDTA

100mM Potassium Phosphate (pH 7.5)

500 $\mu$ M DTTMobility gel mix

30% Acrylamide Mix (Protogel, Geneflow) 14 ml

Glycerol 1.4 ml

5 x TBE 3.5 ml

Sterile distilled water 51.1 ml

10% APS 400  $\mu$ lTEMED 70  $\mu$ l5 x TBE

Tris-HCl 54 g

Boric acid 27.5 g

0.5M EDTA (pH8.0) 20 ml

Sterile distilled water to 1L

2 x Loading dye (10 ml)

1% bromophenol blue 1 ml

50% glycerol 6 ml

1M Tris (pH 8.0) 100  $\mu$ l0.5M EDTA (pH 8.0) 20  $\mu$ l

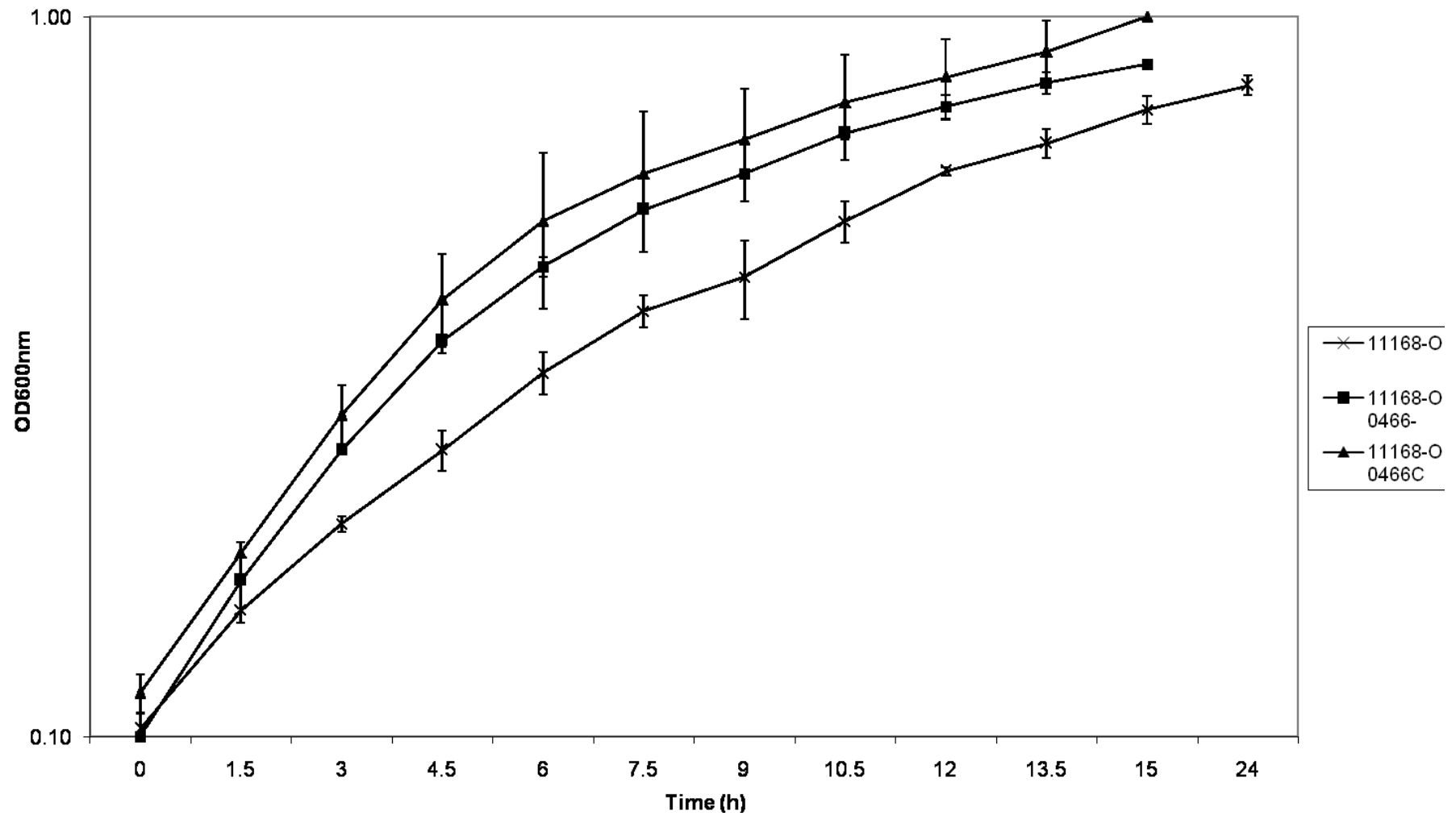
Sterile distilled water 2.88 ml



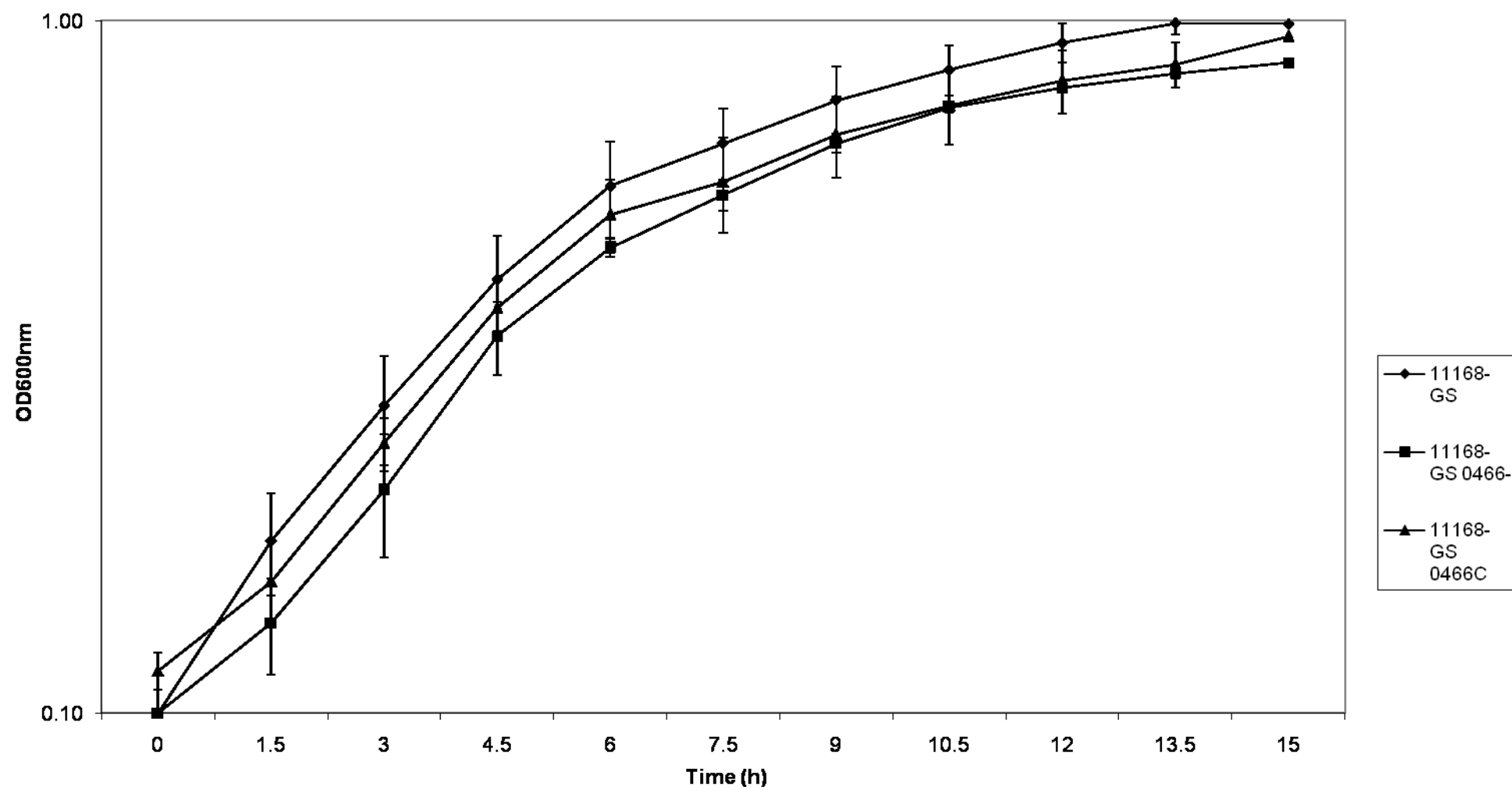
## Appendix III - *Campylobacter* growth data

To determine whether there were any differences in the growth rates of the strains used here, growth curves were constructed for all strains. Muller-Hinton broth was inoculated with the appropriate strain and grown overnight at 37°C in a microaerobic atmosphere containing 5% (v/v) O<sub>2</sub>, 10% (v/v) CO<sub>2</sub> and 85% (v/v) N<sub>2</sub>, with shaking at ~220 rpm. The following day 50 ml of pre-warmed Muller-Hinton broth was inoculated with the overnight culture to an OD<sub>600nm</sub> of 0.1 and grown for 24h. Readings of the OD<sub>600nm</sub> were taken every 1.5h for the first 15h and finally at 24h. Measurements for all strains were repeated three times and the data combined to generate the graphs shown below.

Growth of original background strains in Muller-Hinton broth



Growth of genome sequenced background strains in Muller-Hinton broth



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